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CORRECTION

On page 308, line 1, Volume 39, No. 3, June 1952, read 20 g. for 2 g.

On page 66, line 34, the left column, Volume 39, No. 5 (*Proceedings*), November 1952, read " is formed from " for " is not formed from."

On page 176, line 24, Volume 40, No. 2, March 1953 put the missed lines (underscored) as follows: .. was adsorbed by japanese clay. After the decoloration, the reaction mixture was ..

Misprints in Volume 40, Nos. 1 and 2 should be corrected as shown in the following table:

page	line	correct	wrong
50	13	1.1 moles	1.8 moles
"	19	0.9 moles	1.4 moles
86	12	glass-wool	glass-well
89	Fig. 4, line 1	Influences	Influence
90	26	phosphate	phosphosphate
91	1	is not merely	is merely
"	7	The O_2-uptake	The of O_2-uptake
93	Table IV, Column 5	Metachromatic	Metacromatic
"	Foot note, line 3	there	theae
175	the last line	several	seral
"	Foot note, line 7	extracts	extract
176	8	far	for
183	Fig. 10, line 3	$Nb\text{-}H_2$	$Mb\text{-}H_2$

The Journal of Biochemistry, Vol. 40. ,No. 3.

DEHYDROGENASE PATTERN IN BEAN SEED EMBRYO*

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(Received for publication, February 3, 1953)

A bean seed embryo may be divided into two parts of functional differentiation, i.e. the anabolic part which is morphologically identical with the seedling part including young leaf (plumule), young stem (epi- and hypocotyls), and young root (radicle), and the catabolic one, namely cotyledon.

Though numerous investigations have been made of the redox phenomena in seed embryos of a variety of higher plants, no attempt has yet been made as far as the present authors are aware of to clarify the respiratory pattern in the embryonic tissues corresponding to the above-mentioned functional differentiation.

The present paper which deals with the dehydrogenase activities of the embryonic organs of a legume, *Vigna sesquipedalis*, reveals that every component dehydrogenases of the tricarboxylic acid cycle (the TCA-cycle) except citric dehydrogenase are present both in the anabolic and in the catabolic parts. A categorical difference between these two parts is found to lie in that the distribution of citric dehydrogenase is confined in the anabolic, and the catabolic is entirely deprived of it. It thus seems to be improbable that the TCA-cycle can function in the cotyledon tissue. The lack of the citric enzyme activity in cotyledon may be attributed to the absence of triphosphopyridine nucleotide (TPN), coenzyme of citiric dehydrogenase, in the tissue, where the apoenzyme itself can be detected. The cotyledon tissue appears to be incapable of producing TPN owing to its lower concentration level of energy-rich phosphate bond (~ph) compounds. This, in turn, may

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* Aided in part by the Governmental Research Fund for Science.

* A part of this article was presented before the Annual Meeting of the Botanical Society of Japan held in Tokyo on Nov. 19. 1950, and the compendium of this work was announced at the Annual Meeting of the Botanical Society of Japan held in Tokyo on Oct. 11. 1952.

presumably be caused by the absence of the TCA-cycle, metabolic dynamo perhaps of the highest efficiency, in cotyledon.

It is also reported in this paper that the mechanism of dehydrogenase activation in the germinating seedling may be dualistic in nature, *i.e.* activation of apoenzyme, which will be carried out by conversion of preexisting inert protein into active one, and that of coenzyme, which is presumed to be produced under the energy coupling with the respiratory activity of the tissue.

MATERIALS AND METHODS

Bean Seeds—Seeds of *Vigna sesquipedalis* stored in a dessicator for several months after harvest were used throughout the study. The seeds were soaked in tap water for 5 hours at 30°, then set out on moist sea sand in china pots and grown in the dark at 30°. Preliminary trials had shown that surface sterilization of the seed was unnecessary. The pots and sand, however, were sterilized in flames after they were thoroughly rinsed with tap water.

For the study of the dehydrogenase activity changes in the germination stage, an appropriate number of young plants were harvested at 1-day interval for 6 days of cultivation. As 0-day old materials dry seeds not presoaked were employed. By and large, the growth rate of the seedling as a whole comes down to zero at about the 6th day of cultivation,* it will hence be convenient to call hereafter this 6-day period by the name of the germination stage. Plantlets harvested were separated into four organs each, plumules, hypocotyls, radicles, and cotyledons, by means of a razor blade. Epicotyls were remained being not employed in the present study. Petioles, shoot apices, and seed coats were also discarded. After well washed with distilled water, each organ was homogenized in an ice-chilled mortar** with an adequate amount of cold water. The homogenates were squeezed through cotton cloth, then centrifuged lightly to remove cell-debris, starch grains and other large particles. The resultant supernatants containing most cytoplasmic granules but practically not a cell were employed as the enzyme preparations. The extracts were adjusted to pH 7.4 with NaOH before use.

No attempt to hunger the tissues was previously made. It will, however, be admitted that every embryonic tissues would be consuming in the later period of the germination stage, when the cotyledon tissue is rapidly exhausting its reserve foodstuffs.

The materials for the study described in the section on the mechanism of enzyme activation were prepared as follows: the seedling parts of the dry seed embryos were removed from cotyledons with a razor blade carefully not to be contaminated with the cotyledonous tissue, then sterilized by soaking them in a 60 per cent alcohol, rinsed in distilled water and floated on incubation media in Petri dishes. They were allowed to stand in the dark at 35° for required periods. All the procedures were operated under sterile conditions. Plants were harvested at the times indicated in the figure and the table, then tissue extracts were made from about 250 embryos by the method similar to that above mentioned.

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* The cotyledon-fall begins as early as the 5th day of cultivation.

** Though initially conventional glass-homogenizers were utilized, porcelain mortars were found to be equally satisfactory.

Diphosphopyridine Nucleotide (DPN)—Crude DPN was made from brewer's yeast according to Williamson and Green (*1*).

Triphosphopyridine Nucleotide (TPN)—The crude preparation of DPN (800γ/ml. of final concentration) was substituted for TPN. With preliminary trials with the yeast glutamic dehydrogenase system the DPN preparation had been found to serve also as TPN at the concentration employed.

Adenosine Triphosphate (ATP)—The method of Szent-Györgyi (*2*) was employed using fresh skeletal muscle of a dog $MgSO_4$-injected (*3*).

Reagents—Sodium pyruvate was prepared by the method described in Umbreit *et al.* (*4*), oxalacetic acid by the method described in Yamaguchi (*5*), and α-ketoglutaric acid by the method described in Umbreit *et al.* above-cited (*6*). Other reagents used were purchased in a market. Free organic acids were neutralized by the required amounts of NaOH prior to use.

Yeast Glutamic Apodehydrogenase (*7*)—To Lebedew juice, 1/2 volume of aceton was added, the precipitate was centrifuged off. The resultant supernatant was saturated with CO_2 at O°. Then another 1/2 volume of cold acetone was added to it, and the resultant precipitate was dialyzed over night in a refrigerator against distilled water. The dialysate was employed as the apoenzyme.

Estimation of Dehydrogenase Activity—The conventional Thunberg method, using methylene blue (Mb), was employed. By the aid of a color standard, the time for approximately two thirds decolorization of the dye added was estimated. In most cases, dehydrogenase activity was expressed in terms of reduction rate, definition of which will be given in the text. The color standard was made of 0.3 ml. of Mb (1:10,000), 1.0 ml. of heat-inactivated tissue extract (allowed to stand at 80° for 10 minutes), 2.0 ml. of phosphate buffer (*M*/5, pH 7.4) and 1.7 ml. of water. Further experimental conditions should be consulted to the description of respective figures and tables in the text.

Estimation of Dry Weight—Tissue extract was dried at $100\pm5°$ until constant dry weight was attained. The dry matter was cooled in a silicagel dessicator before weighing.

Estimation of Nitrogen—Total nitrogen was assayed by the method of hypobromite-iodometry of Levy and Palmer modified by Yagi (*8*).

RESULTS

Dehydrogenase Activity Changes in Germination Stage—In the first experiment the changes of various dehydrogenase activities in each embryonic organ during the germination stage were investigated. Nineteen compounds were examined in all. Of these, formic, D-glutamic, malic, succinic, α-ketoglutaric, oxalacetic, pyruvic, glycerophosphoric acids, glucose, and ethyl alcohol were found to be oxidized by the extracts retardation of the endogenous of every organ, while fumaric, lactic, tartaric, oxalic, acetic acids, glycine, glycerine, and vaniline were attacked by none of the organ extracts, sometimes being even inhibitive on the endogenous reducibility of the tissue preparations. Noticeable fact was that the citric dehydrogenase activity was detected only in the anabolic organs, but not in the catabolic one. Citrate added to the cotyledon extract resulted in considerable Mb reduction*. The results of a typical run are shown in Figs. 1–4, in which the data relate to the compounds without or with only week promoting effect on the reduction rate were not described. The term 'reduction rate' which is written on the ordinate of these figures and will be used hereafter in this paper, signifies the rate of Mb-decolorization accelerated by the substrate addition expressed on dry weight basis. It was calculated from the following formula: $100\,(1/t-1/t')/W$, where t is the decolorization time (in minutes)

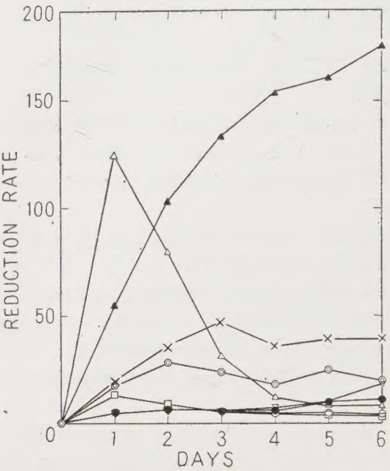

FIG. 1. The changes of dehydrogenase activities of plumules in the germination stage.

Standard system—Tissue extract, 1 ml. (dry weight: about 30 mg.); substrate ($M/10$); 1 ml.; phosphate buffer ($M/5$, pH 7.4), 2 ml.; Mb (1:10,000), 1 ml. Final volume: 5 ml. Substrate and Mb were tipped from the side bulb into main chamber at time zero after temperature equilibration for 10 minutes.

Thunberg tubes; 30°.

—▲—▲—	Citrate
—△—△—	α–Ketoglutarate
—×—×—	Maltate
—◎—◎—	D–Glutamate
—□—□—	Pyruvate
—▽—▽—	Succinate
—●—●—	Formate
—○—○—	Alcohol

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* The citrate inhibition on endogenous Mb reducibility of the cotyledon extract appears to be completely vanished by $(NH_4)_2SO_4$-treatment of the preparation (*cf.* Table III).

in the presence of added substrate, t', the endogenous decolorization time (in minutes), and W, dry weight of the tissue extract employed (in mg.); it was provisionally multiplied by a factor of 100.

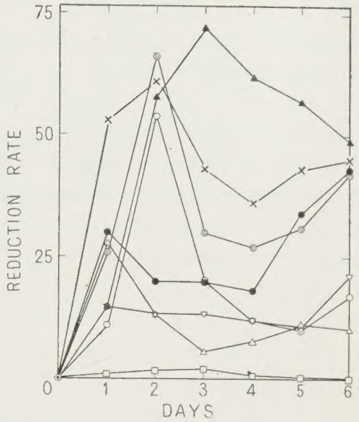

FIG. 2. The changes of dehydrogenase activities of hypocotyls in the germination stage.

Standard system—See the description of Fig. 1.

Thunberg tubes. 30°.

—△—△—	Citrate
—◎—◎—	D-Glutamate
—×—×—	Malate
—○—○—	Alcohol
—●—●—	Formate
—△—△—	α-Ketoglutarate
—▽—▽—	Succinate
—□—□—	Pyruvate

In these figures a prevailing feature is shown concerning the mode of changes of dehydrogenase activities of the developing embryo tissues: that is to say, the activities of every dehydrogenases, except that of citric enzyme of plumule pass *pari passu* their respective peaks in the early germination stage. Passing the peaks, the activities are apt to fall and either finally settle at stable levels as in the case of anabolic organs or continue to diminish until they disappear completely as in the case of catabolic organ. It will be natural that all of the dehydrogenase activities in the cotyledon tissue decay rapidly in the later germination stage, when the tissue withering proceeds. As to the mechanism of the activity fall in the anabolic tissues in the later half of the germination stage, little data are as yet available. It is also obscure why only the citric enzyme activity in plumules does not cease to rise up throughout the germination stage. The mechanism of dehydrogenase activation in the earlier germination stage will be dealt with in the next section.

Following attempts to demonstrate the citric dehydrogenase activity in the cotyledon tissue have been made, but were proved to be in vain: extraction in the presence of a protecting agent such as egg albumin or gelatin which had been recommended in the preparation of unstable pea succinic dehydrogenase (*9*); employment of veronal buffer in place of phosphate buffer which may be poisonous to the enzyme as was reported for liver citric enzyme (*10*); replacement of Mb with thionine of higher redox potential. Negative results in these trials of detection lead us to conclude that the cotyledon tissue is lacking in citric enzyme not in appearance but in reality. It should thus be stressed that only the anabolic organs, but not the catabolic one, of the embryo are provided with every component dehydrogenases of the TCA-cycle, *i.e.* pyruvic, citric, α-ketoglutaric, succinic, and malic dehydrogenases. It is noticeable that the enzyme in question is the only dehydrogenase which is exclusively TPN-specific (*11*) among the TCA-cycle components, and it will be illustrated later that the TPN absence in cotyledon may in

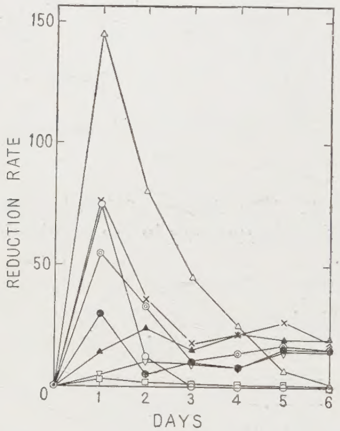

FIG. 3. The changes of dehydrogenase activities of radicles in the germination stage.

Standard system—See the description of Fig. 1.

Thunberg tubes. 30°.

—▲—▲—	Citrate
—△—△—	α-Ketoglutarate
—×—×—	Malate
—◎—◎—	D-Glutamate
—□—□—	Pyruvate
—▽—▽—	Succinate
—●—●—	Formate
—○—○—	Alcohal

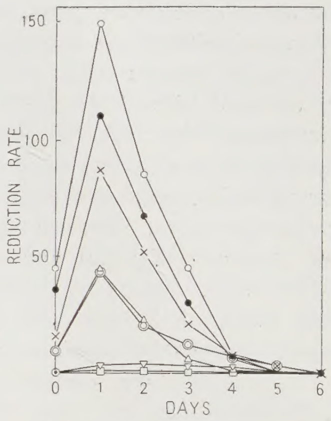

FIG. 4. The changes of dehydrogenase activities of cotyledons in the germination stage.

Standard system—See the description of Fig. 1.

Thunberg tubes. 30°.

—○—○—	Alcohol
—●—●—	Formate
—×—×—	Malate
—△—△—	α-Ketoglutarate
—◎—◎—	D-Glutamate
—▽—▽—	Succinate
—□—□—	Pyruvate

fact be responsible for the absence of the citric enzyme activity in the tissue.

Mechanism of Dehydrogenase Activation—It has been shown above that abrupt rises in the embryonic dehydrogenase activities take place simultaneoulsy as the seeds begin to germinate (Figs. 1–4). The accompanying table (Table I) shows how the protein nitrogen content in the dry matter of respective tissue extracts used changes its value in the germination stage. Separate aliquots of each tissue extract were employed in respective estimations of protein nitrogen and dry weight. As evident from the table, protein content of respective tissue preparations remains practically uniform throughout the germination stage in comparison with the remarkable changes in dehydrogenase activities of the same preparations. Hence it may be assumed that the changes in the dehydrogenase activities do take place with little concomitant changes in the total protein contents of the preaparations. There will be no need to say at this place of the cotyledonous dehydrogenases, the activities of which begin to rise to considerable heights immediately after the start of germination, though any anabolic work including naturally that of enzyme protein synthesis would not be done there. Furthermore, it will also be interesting to see that the embryos artificially decotyledonized, and, therefore, practically deprived of anabolic function

TABLE I

Changes of Protein Nitrogen Contents (in per cent) in the Dry Matters of Various Tissue Extracts Employed for the Dehydrogenase Estimation Illustrated in Figs. 1–4

Organ	Age of tisue (*days*)			
	1	2	3	5
Plumules	5	6	7	7
Hypocotyls	3	2	2	1
Radicles	3	3	3	4
Cotyledons	9	8	6	5

The tissue extracts were treated first with cold and next with hot trichloracetic acid (final concentration: 10 per cent) to remove acid soluble compounds as well as nucleic acids. The total nitrogen content of the residual precipitation was estimated as the protein nitrogen.

due to the cessation of the raw material supply from the reservoir, appear to be yet able to increase their apoenzyme contents as only the tissues swell (Fig 5, Curve B).

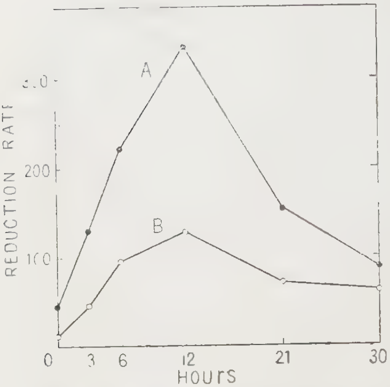

Fig. 5. The change of the malic dehydrogenase activity of decotyledonized embryos floated on water.

Standard system—Tissue extract, 1 ml. (dry weight: about 30 mg.); DPN (2 mg./ml), 1 ml.; malate (*M*/10), 1 ml.; phosphate buffer (*M*/5, pH 7.4), 1 ml.; Mb (1:10,000), 1 ml. Final volume: 5 ml. Curve A: With DPN; Curve B: Without DPN.

Thunberg tubes. 30°.

Fig. 5 illustrates the malic dehydrogenase activity of the decotyledonized embryos floated for various periods on distilled water* in the dark at 35°. After the designated times, the dehydrogenase activity of the tissue extracts was determined in the presence (Curve A) as well as in the absence (Curve B) of an excess of DPN added. It had preliminarily been confirmed that the enzyme was saturated with the DPN concentration employed. Curve A would enable one to guess the mode of change in the potential amount of the malic apoenzyme per dry weight of the tissue extract. Hence, its characteristic shape with a peak similar to that observed for the intact embryos would indicate that the amount of apoenzyme can be increased as the tissue only swells in water. And as will be discussed below, the peak here attained is higher than that attained by intact embryos. Hence, it may be thought that the apoenzyme production in the early germination stage may practically be independent of the cotyledonous reserve. Here also occurred the activity fall after the peak was passed, the mechanism of which is as yet obscure.

The factor limiting Curve B may be the coenzyme content in the preparation; if saturating amount of the coenzyme is available, Curve B should be raised up to the level of Curve A. It may thus be clear from Curve B that the change of coenzyme content just keeps pace with that of apoenzyme content. It has to be noted that this change of the coenzyme content in the seedling takes place intrincically, in other words, independently of the supply of reserves from cotyledon.

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* Results of a similar nature were obtained with respect to the seedling glutamic dehydrogenase. Details of the experiment are not presented here.

By the way, in the case of the decotyledonized embryos the highest peak of the reduction rate was attained far sooner (within a half day incubation) and, furthermore, was much higher (reduction rate of about 130) than in the case of intact embryos (the peak at most 70 in height was attained only on the 2nd day of incubation. (*cf.* Figs. 2, 3). The reason for the former fact may be that the artificial removal of cotyledon necessarily brings the whole seedling tissues sooner in contact with not only water but also oxygen, and, thus, favors the enhancement of the enzyme activity. It is widely accepted that the aerobiosis is of special significance in the enzyme activation of the vegetable embryos (*cf.* 12). No satisfactory explanation, however, can at present be offered to account for how the dehydrogenase activity of the decotyledonized embryos could rise considerably higher than that of the intact embryos do.

In an attempt to gain some indication concerning the energy relations of the production of apo- and coenzymes in the embryonic tissues, the effect of 2,4-dinitrophenol (2,4-DNP), so-called ‘uncoupling agent,’ on these production processes was investigated.

Decotyledonized embryos were floated on distilled water and on a 2, 4-DNP solution (10^{-5} *M*), respectively. After 8 hour incubation

TABLE II

The Effect of 2,4-DNP on the Malic Dehydrogenase Production in the Decotyledonized Embryos

Culture media	Water		2,4-DNP (10^{-5} *M*)*	
DPN addition	—	+	—	+
Reduction rate	88.7	148	55.5	118
2, 4-DNP inhibition (*per cent*)			38	20

Standard system—Tissue extract, 1.0 ml. (dry weight: about 35 mg.); DPN (2 mg./ml.), 1 ml.; malate (*M*/10), 1 ml.; phosphate buffer (*M*/5, pH 7.4), 1 ml.; Mb (1:10,000), 1 ml. Final volume: 5 ml. Thunberg tubes. 30°.

* 2,4-DNP at the concentration used was found to be incapable of decreasing but rather slightly accelerate the respiration and much enhanced the aerobic fermentation of the embryonic tissues.

in the dark at 35°, the embryos were withdrawn from the media and minced with about 7 ml. of water, respectively. The malic dehydrogenase activities of these extracts were examined in the presence as well as in the absence of a saturating concentration of DPN. A typical result is shown in Table II. It has already been mentioned that the activity estimated in the presence of an excess amount of DPN would denote the apoenzyme quantity, and that estimated in the absence of added DPN the coenzyme quantity. It is thus evident from this table that 2,4-DNP can prevent the enhancement of both apoenzyme and coenzyme, and that the process of coenzyme production is more sensitive to the action of 2,4-DNP than that of apoenzyme production is (38 per cent inhibition of coenzyme activation *vs.* 20 per cent inhibition of apoenzyme activation, respectively caused by 10^{-5} M 2,4-DNP).

En passant, the possibility will not be excluded here that TPN-specific ' malic enzyme ' (*13*) has a share in the malic dehydrogenation under consideration. Even if it is the case, the above described as to the activation process of malic dehydrogenase would not be needed to be much revised, and only the word ' coenzyme ' in these paragraphs should be read as coenzymes including both DPN and TPN. It will reasonably be supposed that DPN added to the seedling tissues would at least partially be converted into TPN (See the later description).

Distribution of Citric Apoenzyme in Cotyledon—It has been shown that the cotyledon extract *per se* had no activity of citric dehydrogenase. Nevertheless, in the presence of TPN added the extract was disclosed to be able to dehydrogenize citiric acid.

TABLE III

The Effect of TPN on the Citric Dehydrogenase Action of the Crude Protein from Cotyledon Two Days Old

Cotyledon protein	+	+	+	+
TPN	−	−	+	+
Citrate	−	+	−	+
Mb reduction time (*minutes*)	260	260	81	58

Standard system—Crude cotyledon protein, 1 ml.; TPN (4 mg./ml. of DPN, instead), 1 ml. citrate (M/10), 1 ml.; phosphate buffer (M/15, pH 7.8), 1 ml.; Mb (1:10,000), 1 ml. Final volume: 5 ml. Thunberg tubes. 30°

Cotyledons removed from 2 days old 200 plants were well minced with 30 ml. of water, then equal volume of saturated $(NH_4)_2SO_4$ solution was added. Resultant precipitate was employed as the apoenzyme without subsequent dialysis to remove any accompanying $(NH_4)_2SO_4$. A typical result obtained is shown in Table III.

The endogenous Mb reduction rate was promoted by the addition of TPN (Column 3). This may be attributed to the possible contamination of the TPN preparation with compounds which can be attacked by dehydrogenases in the crude protein preparation. Further addition of citrate, which by itself had no influence on the endogenous reduction rate of the enzyme preparation (Column 2), caused evident shortening of the reduction time (Column 4). This indicates the existence of citric apoenzyme in the cotyledon preparation, which in collaboration with TPN added can function as the holodehydrogenase.

Distribution of TPN in Embryonic Tissues—Water extracts of cotyledons as well as seedlings were investigated with regard to their TPN action. Two days old 200 plants were separated into these two parts. Respective parts were homogenized with an adequate amount of water, then squeezed through cotton cloth. The water extracts, after concentrated over a water-bath, were washed several times with ether to remove endogenous substrates as much as possible. The remaining ether was driven away on a water-bath. Flocculent precipitates were filtered off. The filtrates (respectively about 5 ml. in all) were examined whether or not they were effective as TPN in the yeast glutamic dehydrogenase system. A typical result is shown in Table IV. The addition of tissue extract caused considerable shortening of the endogenous reduction time probably due to remaining substrate contamination.

TABLE IV

TPN Action of the Cotyledon as well as the Seedling Extracts

Yeast glutamic apoenzyme	+	+	+	+	+	+
Cotyledon extract	−	−	+	+	−	−
Seedling extract	−	−	−	−	+	+
Glutamate	−	+	−	+	−	+
Mb reduction time (*minutes*)	80	80	18	18	30	19

Standard system—Yeast apoenzyme, 1 ml.; glutamate (*M*/10), 1 ml; tissue extract, 1 ml.; Mb (1:10,000), 1 ml.; phosphate buffer (*M*/15, pH 7.8), 1 ml. Final volume: 5 ml.

Thunberg tubes. 30°.

Further addition of glutamate was found to be effective only in the qresence of the seedling extract. The cofactor for the yeast apoenzyme (TPN) is thus supposed to be distributed exclusively in the seedling part but not in the cotyledon one.

Considering the facts described earlier that the cotyledon tissue is rich in formic, malic*, and glutamic dehydrogenases, all known to be DPN linked (*cf. 14*), the existence of DPN in cotyledon will be in little doubt. It appears to have been established that TPN will be synthesized *in vivo* as well as *in vitro* by phosphorylation of DPN (*15*), and, hence, the TPN absence in cotyledon may, in all likelihood, be attributed to the incapability of this tissue of the DPN phosphorylation action. This supposition was confirmed inducing the TPN action in the cotyledon preparation with the aid of $\sim$ph donor added. That is, crude protein from cotyledon similar to that employed in the above experiment (Table III) was previously exposed to ATP for an hour, and thereafter the citric dehydrogenase action of the preparation was examined. The result is shown in Table V.

It has been pointed out that the cotyledon protein employed could

TABLE V

The Effect of ATP on the Citric Enzyme Action of the Crude Cotyledon Protein

Cotyledon protein	+	+	+	+	+	+
ATP	−	−	+	+	+	+
Citrate	−	+	−	+	−	+
ATP addition			at the start of the Mb test		60 min. before the start of the Mb test*	
Mb reduction time (*minutes*)	90	90	36	36	26	20

Standard system—Crude cotyledon protein, with phosphate buffer ($M/20$, pH 7.0) and $MgCl_2$ ($M/150$), 1 ml.; ATP ($M/65$), 1 ml.; citrate ($M/10$), 1 ml.; Mb (1:10,000), 1 ml.; water, 1 ml. Final volume: 5 ml.

Thunberg tubes. 30°.

* Crude protein solution (1 ml.) was exposed to ATP ($M/65$, 1 ml.); hence, the concentration of ATP in the preincubation period was $M/130$.

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* Because of the fact that practically no TPN is present in cotyledon, the 'malic enzyme' would have nothing to do with the malate oxidation in this tissue.

show the citric dehydrogenase action in the presence of TPN added, and the authors' separate experiments, in which the formic enzyme activity was demonstrated by the Thunberg technique with the protein preparation in question, suggests the existence of DPN in this crude protein. Furthermore, it is clear from the result shown in Table V (Columns 3, 4) that the ATP preparation employed is practically contaminated with no TPN. Hence, the data shown in Columns 5,6 would indicate that the pretreatment of the protein preparation with ATP is effective in converting preexisting DPN into TPN. An interesting supposition may thus be introduced here: the shortage in, or the low level of ~ph compounds in the cotyledon tissue would probably be responsible for the TPN absence under consideration.

The shortening of the control reduction time brought forth by the ATP addition could possibly be ascribed to one or both of following factors: (a) contamination of this crude ATP preparation with unknown various dehydrogenase substrates, and (b) promotion of the DPN production by ATP added (*16*). It will be natural to expect that the crude protein employed may have various apodehydrogenases other than citric apoenzyme.

DISCUSSION

The foregoing experiments suggest a possibility that there exists a wide difference in efficiency between the respiratory mechanism of the anabolic organs and that of the catabolic one of the bean seed embryos. Exclusively the former can be provided with the TCA-cycle, metabolic dynamo probably of the highest efficiency ever known, while in the latter the cycle cannot function due to a missing link, citric dehydrogenase. This possible lower efficiency of the exergonic function in the catabolic tissue would mean its lower efficiency in ~ph production, and the resultant lower ~ph level in the tissue may be reflected in the very catabolic nature of the tissue, where practically any synthetic process, inclusive of TPN production, may not take place. The absence of TPN, in turn, can hinder the TCA-cycle from functioning because of the stoppage of the action of a component enzyme, citric dehydrogenase, which needs specific collaboration of TPN in its action.

It is noticed that the word, lower ~ph level in the catabolic organ, namely in cotyledon, does not signify the absence of ~ph compounds in the tissue. As now well established, most common degradation processes of high molecular compounds such as starch or protein, of which the bulk of the cotyledonous reserves is consisted, involve intermediate steps of phosphorylation with the participation of ~ph donors as ATP. What is then the threshold ~ph level that is decisive of the anabolic or catabolic characters of the embryonic tissues? In this respect, it must be remembered that cotyledon, which is entirely catabolic at least in its fully developed state, had once to be highly anabolic, too, when it was not only to grow its tissue itself but also to produce its reserve foodstuffs in the earlier period of seed maturation on the ovule placenta. Generally speaking, the anabolic functions should be coupled energetically with catabolic ones. So it may be of special interest to explore when and how in the maturation process of cotyledon its anabolic abilities dissapear leaving merely the catabolic ones. Direct method of approach may be to examine the time course of the change of metabolic pattern in the maturation process of the cotyledon tissue.

It should be mentioned here an interesting finding of the TPN destroying factor by Vennesland and co-workers (*13*). According to these workers, the action of this factor which is believed to be of widespread distribution in plant tissues can be prevented by the addition of ATP, DPN, or muscle adenylic acid. The possibility, therefore, seems to be not excluded that the apparent promoting effect of ATP on the TPN production in the cotyledon protein preparation (*cf.* Table V), is, interpreted as merely the protection effect of ATP against the enzymatic decomposition of TPN which was originally present in cotyledon. However, separate experiments conducted by the present authors showed that the crude protein from cotyledon was not able to have the TPN action as long as it was ' protected ' with DPN at final concentrations less than 400 γ/ml. And, moreover, as the present experiment (Table V) indicates, the ATP effect of the ' TPN protection ' could not be realized unless the protein was previously exposed for some duration of time to the ~ph donor. Considering these facts together, the abovementioned interpretation that in this case the ATP effect is top romote the TPN production but not to protect the TPN destruction seems to be valid. This point, however, would require additional investigations.

As for the activation mechanism of dehydrogenases in the germinating embryos, the investigations on malic (as well as glutamic) dehydrogenase reveal that the production of apoenzymes (protein parts of the enzymes) may be accompanied with no protein synthesis and is less sensitive to the uncoupling action of 2,4-DNP than the production of coenzyme is. These facts would suggest the dualistic nature of the dehydrogenase activation mechanism: activation of apoenzymes which may involve some conversion of inert proteins into active ones under the influence of, say, hydrolytic enzymes and would be less intimately associated with the exergonic functions of the tissues; on the other hand, the activation of coenzymes which may involve syntheses of the coenzymes themselves under the energy supply from the respiratory functions of the tissues.

SUMMARY

1. Dehydrogenase activities of tissue extracts of embryonic organs of a legume, *Vigna sesquipedalis*, germinated and grown in the dark wholly at the expense of its own reserve foodstuffs were investigated by the Thunberg method.

2. Of 19 compounds tested, 10, including every component acids of the TCA-cycle, *i.e.* pyruvic, citric, α-ketoglutaric, succinic and malic acids, were oxidized under the anaerobic conditions by each anabolic organs investigated, namely, plumules, hypocotyls, and radicles. On the other hand, the catabolic organ, cotyledon, was found to have the same dehydrogenase pattern as observed in the anabolic ones but for the absence of citric dehydrogenase, the only TPN-linked enzyme in the TCA-cycle.

3. TPN was detected in all anabolic organs tested but not in the catabolic one, while DPN was found to be distributed ubiquitously in the embryonic tissues.

4. Addition of either TPN or ATP to crude protein from cotyledon could evoke the citric dehydrogenase action. In the case of ATP experiment, it was required to incubate previously the preparation for a short time with ATP so as to induce the enzyme activity. DPN preexisting in the protein preparations was presumably to be phosphorylated into TPN in this incubation period.

5. Every dehydrogenase activities of embryonic tissues expressed on a dry weight basis rose rapidly as the seed germinated and most of them soon passed their respective peaks. However, the protein contents in a unit dry weight of the preparations showed little change in the germination stage. Thus the enzyme activations have practically not to involve syntheses of new enzyme proteins, but probably hydrolytic conversion of some inert proteins into active ones.

6. Production of both apoenzyme and coenzyme of malic dehydrogenase was inhibited by 2,4-DNP. This uncoupling agent affected the process of coenzyme activation more strikingly than that of apoenzyme activation. Hence, aerobiosis is thought to be more necessary conditions for the activation of coenzyme than for that of apoenzyme.

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STUDIES ON THE REACTION PROCESS OF CHOLEGLOBIN FORMATION

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Choleglobin, one of the green derivatives of hemoglobin, is formed as the result of oxidative disintegration of hemoglobin molecule in the reaction system of hemoglobin, ascorbic acid and oxygen. Though the process of this reaction has long been the subject of several investigators (*1*, *2*, *3*, *4*), the essential nature of the reaction mechanism remains as yet unclarified in many points.

The main object of the present work is to make better and more extensive analyses of the process of the choleglobin formation. Besides, on the basis of the results obtained from our experiments, a hypothetical reaction scheme for the mentioned reaction system is presented.

EXPERIMENTAL METHODS

The red cells repeatedly washed with physiological saline solution were laked by means of dilution with redistilled water, and centrifuged. The supernatant blood pigment solution was used as the starting material in its 0.6 per cent solution. Ascorbic acid was dissolved in redistilled water and neutralized with NaOH; the solution was prepared every time immediately before use.

The experiments were carried out as follows: the reaction solution of a definite composition, required in each experimental condition, was brought into a number of beakers of the same size and capacity and were kept at desired temperature. The conditions of each experiment will be described in each series of experiment in detail.

The present experiment consisted in following estimations:

(a) Estimation of choleglobin (choleheme) formed in the reaction mixture by means of spectrophotometry. The method is described in the next section. (b) Consumption of ascorbic acid was followed up by the following procedure: the reaction solution was deproteinized with acetic acid and sulfosalicylic acid and its ascorbic acid content was titrated with KIO_3 after the addition of KI. The concentration of KIO_3 used for the titration was held at $N/80$—$N/10$ in respect to that of ascorbate. (c) Liberation of iron: to split out the iron atom from choleheme the method of Barkan (*5*) was used in principle. For the quantitative determination of the splitted iron, o-phenanthroline method of Barkan (*6*) was modified in some points.

RESULTS

I. A Method for the Quantitative Determination of Choleglobin Production in the Reaction Process (Experiments by Kikuchi):

The methods hitherto employed by many investigators for the studies of this reaction process are mostly insufficient in the accuracy as demanded for the more detailed study. Thus a more reliable method for the quantitative follow up of the process was planned to be established.

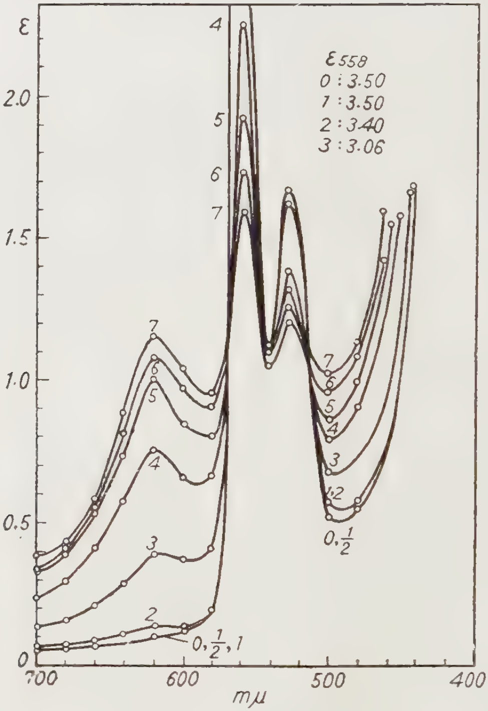


Fig. 1. Changes in the absorpton intensities during the reaction process.

5.0 ml. 0.6% O_2Hb, 4.0 ml. *M*/15 phosphate buffer and 1.0 ml. 17.6 mg./ml. ascorbic acid were mixed. Incubated at 37.5°. After 0,1/2,1,2 . . . 7 hours, each of the reaction solution was reduced by addition of a small amount of $Na_2S_2O_4$ and immediately after that the absorption intensities (440–700 mμ) were measured after the addition of 5 ml. of 3% NaOH to make them clear. With the numbers in the figure, the lengths of the reaction time are given.

A hemoglobin solution obtained from the red cells of rabbit was subjected to be decomposed to form choleglobin under the condition of the reaction medium as described in Fig. 1. When the reacted solution was treated with NaOH and $Na_2S_2O_4$, there appeared a new absorption maximum in the red at 618 mμ (cholehemochrome figure) corresponding to the amount of choleglobin formed. In the green, however, two maxima at 558 and 527 mμ of alkali denatured globin-hemochrome decreased in their optical density. The changes of their densities were plotted against time as indicated by the curve in Fig. 2 (full line).

Since the denaturation of globin molecule occurs in the course of the process and a green precipitation, designated as cholechemochromogen by Lemberg (*1*), is formed, the investigations were made separately in fractions. A portion of reaction solution was taken out at certain intervals of time and was centrifuged to separate the supernatant solution from the precipitate. Both fractions were then separately treated with NaOH and $Na_2S_2O_4$.

With the supernatant solution, the increase in the optical densities at 618 mμ was proportional to the length of the reaction time. The densities at the two maxima in the green, however, decreased continuously as the reaction proceeded.

With the precipitates, however, the optical densities at 618 mμ continued to increase with time until it reaches to the maximum at the end of the reaction. The absorption at 558 mμ remained just traceable in the precipitates. From above facts, the reaction is assumed to proceed in some way as follows: hemoglobin → choleglobin → cholehemochromogen. The chemical changes in the heme part of both choleglobin and cholehemochromogen are supposed to be identical from their absorption property. Then, the following equation can be obtained from the estimated curves given in Fig. 2 (full line).

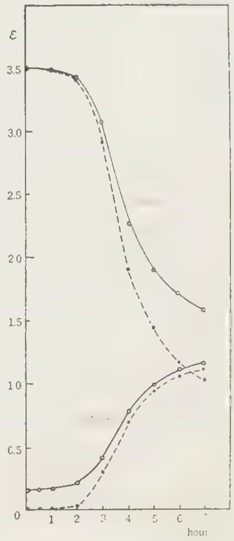


FIG. 2. Changes in the amounts of protoheme remained unaffected and cholehme formed during the reaction.

Full lines: Plotts of ε_{558} and ε_{618} as observed (from Fig. 1) against time. Dotted lines: Plotts of ε_{558} by component x and ε_{618} by component y against time. They are calculated from the equations in the text (next page).

$$\varepsilon_{558} = x + 0.5\, y$$
$$\varepsilon_{618} = y + 0.03\, x$$

where x, ... actual ε_{558} of alkali denatured protohemochrome,
y, ... actual ε_{618} of alkali denatured cholehemochrome,
0.5, ... $\varepsilon_{558}/\varepsilon_{618}$ of alkali cholehemochrome,
0.03, ... $\varepsilon_{618}/\varepsilon_{558}$ of alkali protohemochrome.

Applying these equations to the experimental curves given in Fig. 2 (full line), x and y can be obtained by calculation as shown in Fig. 2 (dotted lines). Thus the amount of remaining protoheme (or its percentage) at any given time of the reaction can be known from Fig. 2 by calculation with the absorption-concentration curve of alkali protohemochrome in the wavelength of 558 mμ, but under the presumption that the choleheme is the only reaction product of the given system. This enables us to determine the amount (or percentage) of choleheme produced in the system by subtraction of remaining amount of protoheme from its initial amount. Now, the amount of choleheme produced were determined by the outlined procedure from the results of several experiments and these were plotted against each of their optical densities ε_{618} observed. The relationship is given in a linear function as shown in Fig. 3. Each point falls on the straight line, especially accurate at the early stadium of the reaction. The identity of the chemical nature of the heme parts of choleglobin and cholehemochromogen is thus proved as assumed by us, and further it is highly probable that no by-reaction occurs in this system.

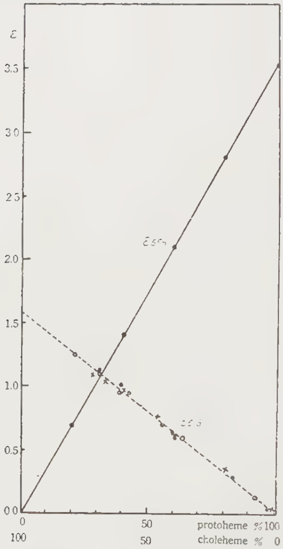


FIG. 3. Calibration curve for the determination of protoheme and choleheme existing in the reaction mixture.

Full line: protoheme in alkali denatured globin-hemochrome. Dotted line: choleheme in alkali denatured globin-cholehemochrome. 100% in the figure corresponds to hemoglobin of 0.2% concentration and to heme of 1.176×10^{-4} *M/lit.* Choleheme is in equivalent with protoheme.

All these show that the presented method enables us to follow up the amount of choleglobin production in the mentioned process with sufficient accuracy and reliability.

In the later stadium of the reaction, however, the points experimentally plotted run underneath the straight line. This seems apparently due to the further changes of the choleheme once produced. Thus the straight part of the line drawn in Fig. 3, can be used as the concentration-absorption curve for the choleheme. In the concentration of alkali protohemochrome corresponding to 0.2 per cent of hemoglobin solution $\varepsilon_{558}=3.5$ and in that of alkali cholehemochrome $\varepsilon_{618}=1.6$ as determined approximately. With the assumption that the molecular weight of both hemoglobin and cholehemochromogen is 67,000, the molar extinction coefficient of choleheme in the molecule of alkali cholehemochrome can be given as $\varepsilon_{\text{mol.}}=1.36\times10^4$.

II. The Relation between the Formation of Choleheme and the Oxidation of Ascorbic Acid (*Experiments by Kikuchi and Ogawa*)*:*

The hemoglobin solution was prepared from human erythrocytes. In the present experiments, a non-catalase hemoglobin solution was further used which was prepared from the red cells of a peculiar patient "*Anenzymia catalasea*" (7) in whom the catalase is deficient.

a) *The Reaction Process in the Absence of Catalase*—The experiments were carried out with non-catalase hemoglobin solution. The formation of choleglobin was studied in the system with varying concentrations of ascorbate. The other conditions in the system were kept constant. The results are given in Fig. 4. The rate of choleheme production increased as the ascorbate concentration increased.

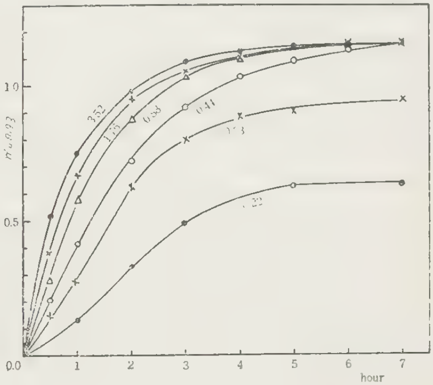


FIG. 4. Time curve for the choleheme formation in the absence of catalase. 5.0 ml. 0.6% O_2Hb solution with 4.0 ml. *M*/15 phosphate buffer (pH 7.38) and 1.0 ml. ascorbate solution (neutralized with NaOH). Temperature, 37.5°. Numbers in the figure represent the final concentrations of ascorbic acid in mg./ml.

The formation of choleheme in the system with its ascorbate concentration of 0.22–0.33 mg./ml. never reached to the maximum value, which shows that the ascorbate added is used up before the reaction has been completed. In the higher concentrations of ascorbate, choleheme was formed immediately from early stadium, while in the relatively lower ascorbate concentrations the initial rate of choleheme formation was greatly depressed. Thus reaction curves of the former show a hyperbolic nature, while those of the latter are S-shaped. In the ascorbate concentration of 0.44 mg./ml. and above, the formation of choleheme reaches almost to its constant limit after 7 hours. In these cases the maximum amount of choleheme formed corresponds to about 75 per cent of the theoretical. About 25 per cent of the protoheme remains thus unaltered. About the same values were obtained also in the experiments with hemoglobin solution of the rabbits (section I).

From the fact that the maximum formation of choleheme occurs only in the concentrations of ascorbate of 0.44 mg./ml. and above, the consumed moles of ascorbate in the process of choleheme production can be calculated as about 20 moles per mole of protoheme (*cf.* also Lemberg).

b) *The Reaction Process in the Presence of Catalase*—The same experiments were carried out with normal human hemoglobin. The results are given in Fig. 5. In these experiments, the formation of choleheme proceeded only with S-shaped curves and this was more significant in relatively lower ascorbate concentrations. As long as the ascorbate concentration was held sufficiently high, however, the alteration of ascorbate concentrations had an effect only on the initiation of the process of choleheme formation, and when the reaction once began, the ascorbate concentration had no more effect on the reaction rate. After that, the reaction proceeded in the same rate showing rapid raise of the curve. They differed from those with non-catalase hemoglobin only in the length of their lag phase.

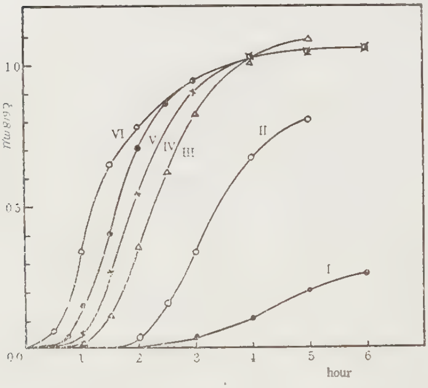


FIG. 5. Time curve for the choleheme formation in the presence of catalase. The reaction conditions: same as described in Fig. 4. Concentrations of ascorbate: I, 0.22, II, 0.44, III, 0.88, IV, 1.76, V, 3.52, and VI, 5.28 mg./ml.

Now, the oxidation of ascorbate was estimated in the system of the same conditions as in the experiments of Fig. 5. As shown by Fig. 6,

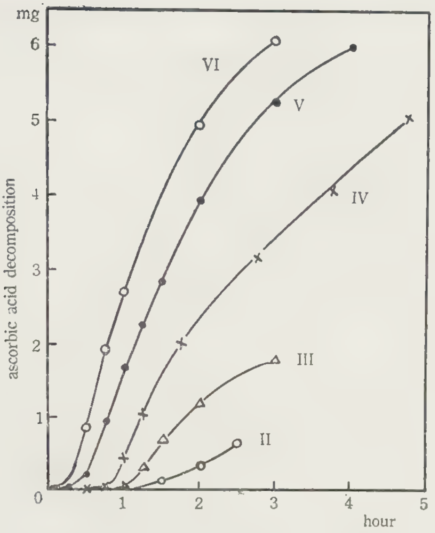


FIG. 6. Time curve for the decomposition of ascorbic acid in the presence of catalase.

The reaction conditions: same as described in Fig. 4. Numbers in the figure represent the same concentrations of ascorbate as in Fig. 5.

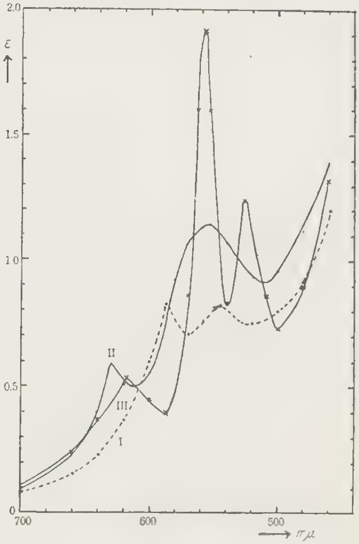


FIG. 7. Absorption figures showing the degradation of Hb by the addition of H_2O_2.

Reaction mixture: 1.0 ml. 0.6% O_2Hb solution, 1.5 ml. $M/15$ phosphate buffer (pH 8.8) and 0.5 ml. $M/30$ H_2O_2.

I. Absorption spectra of the reaction solution immediately after the reaction started (showing the figure of H_2O_2-metHb compound).

II. After the addition of $Na_2S_2O_4$ to the solution I.

III. After the addition of 1.0 ml. 3% NaOH to the solution II (intensities are given by the multiplication of 4/3, corresponding to the dilution).

the oxidation of ascorbate forms a similar S-shaped curve as in the choleheme formation. By comparison of Fig. 5 and 6, it is now clear that the oxidation of ascorbate proceeds rather rapidly already before the choleheme formation begins. The oxidation of ascorbate was accelerated with increasing concentrations of ascorbate as in the case of the choleheme formation which is indicated in Fig. 5.

III. The Reaction of H_2O_2 upon Hemoglobin (Experiments by Kikuchi):

In the reaction system of O_2Hb, ascorbic acid and oxygen, H_2O_2 is generated, which seems to play an essential role in the formation of choleglobin. It seems to be of great interest to study the chemical action of H_2O_2 upon hemoglobin, especially upon hemoglobin without catalase. We have thus studied the reaction of H_2O_2 upon hemoglobin in various concentrations of the former. Under the conditions described in Fig. 7, H_2O_2 was added to O_2Hb solution in the absence of ascorbate; there appeared the absorptions of H_2O_2-metHb-complex (Fig. 7, I). When a small amount of $Na_2S_2O_4$ pulver was then added to the solution, there appeared an absorption figure of reduced hemoglobin in the green, while in the red region there appeared a new absorption maximum at 630 mμ (Fig. 7, II). This wavelength of the maximum at 630 mμ is in agreement with that of choleglobin. When the globin is strongly denatured by the addition of some NaOH to this solution, a displacement of the absorption maxima, 630→618 mμ will then occur. This position of the wavelength is again in agreement with that of alkali cholehemochrome absorption.

From the fact it may be assumed that the product is choleglobin. The yield of this compound in the present reaction system increases as H_2O_2 concentration increases. But in this case some propentdyopent compound is formed simultaneously. The same reaction carried out in the presence of ascorbate, however, revealed that ascorbate had no effect upon this reaction process, in spite of our expectation that it would have (Table I).

TABLE I

1.0 ml. 0.6% O_2Hb, 1.0 ml. *M*/15 phosphate buffer (pH 8.8), 0.5 ml. ascorbic acid, and 0.5 ml. *M*/30 H_2O_2

$Na_2S_2O_4$ was added after 1 minute of the H_2O_2 addition. 30 seconds thereafter, 1.0 ml. of 3% NaOH solution was added

Concentrations of ascorbate added (*mg./ml.*) / Optical density and wavelength (mμ)	0	8.8	17.6	35.2	70.4
558	1.45	1.44	1.40	1.43	1.45
527	0.87	0.86	0.85	0.84	0.87
618	0.38	0.38	0.37	0.38	0.38

In the process of choleglobin formation, some interesting phenomena took place when the order of mixing of the reactants was altered.

i) It was found that when O_2Hb was first reduced by the addition of $Na_2S_2O_4$ and H_2O_2 was added after that, the choleglobin could then also be formed. This indicates that hemoglobin may not necessarily be bound to oxygen to form O_2Hb for the choleglobin formation.

ii) The following three series of reaction were investigated. The mixing of the reagents was made in the following order:

(a) $O_2Hb+H_2O_2+NaOH+Na_2S_2O_4$
(b) $O_2Hb+H_2O_2+Na_2S_2O_4+NaOH$
(c) $O_2Hb+NaOH+H_2O_2+Na_2S_2O_4$

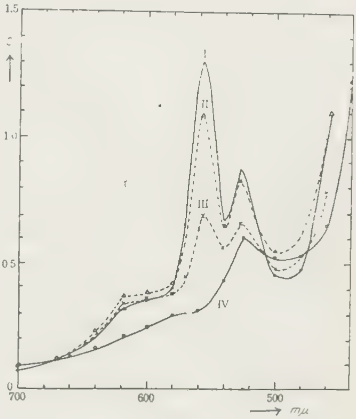


FIG. 8. Absorption figures showing the effect of globin denaturation on the degradation of hemoglobin by H_2O_2; NaOH being added after H_2O_2 but preceeding the addition of $Ha_2S_2O_4$.

Reaction mixture: 1.0 ml. 0.6% O_2Hb solution, 1.5 ml. $M/15$ phosphate buffer (pH 8.8) and 0.5 ml. M/n H_2O_2.

1.0 ml. 3% NaOH was added after 1 minute of the reaction and $Na_2S_2O_4$ was then added after further 30 sec.

Concentrations of H_2O_2: I, $n=200$; II, $n=100$; III, $n=20$; and IV, $n=10$.

Addition of NaOH leads undoubtedly to the denaturation of globin.

The system-(a) leads to greater by-reaction, so that the yield of choleheme and the remaining amount of protoheme are both smaller than in the system-(b). But, as shown in Fig. 8, significant absorption is observed at 600–620 mμ in the a) reaction. The system-(c) leads by no means to choleglobin formation.

These facts indicate that for the process of choleglobin formation by the action of H_2O_2 upon hemoglobin, its globin part should not be denatured too far, and further that the reaction proceeds not through the direct action of $Na_2S_2O_4$, but that the reaction can proceed without the addition of $Na_2S_2O_4$.

iii) A similar set of experiments as above was carried out, this time with methemoglobin instead of hemoglobin. The systems studied and the results are as follows:

(a′) met $Hb+H_2O_2+NaOH+Na_2S_2O_4$ . . choleglobin formation (—)
(b′) met $Hb+H_2O_2+Na_2S_2O_4+NaOH$ . . ,, ,, (+)
(c′) met $Hb+NaOH+H_2O_2+Na_2S_2O_4$ . . ,, ,, (—)

By comparing these reactions with those of O_2Hb, the system-(a′) seems to be of greatest interest. While in the reaction-(a) with O_2Hb, choleglobin was formed in good yield (Fig. 8); in the reaction-(a′) with met Hb, however, no specific absorption is recognisable in the red region—the absorption property after completion of the reaction is qualitatively and quantitatively in fair agreement with that of system c) with hemoglobin.

From above observations the following fact may now be pointed out. For the formation of choleglobin through the action of H_2O_2 upon hemoglobin, its globin part should not be denatured too far and the electric charge of hemoglobin molecule should necessarily be kept in Fe^{++} state. The reaction process of choleglobin formation seems thus to be that played by the peroxidatic activity of heme molecule, its substrate being its own heme molecule which is oxidized by H_2O_2 peroxidatively. The detailed studies on this subject were published in another paper (*8*).

IV. Choleglobin Formation from Methemoglobin as the Starting Substance (Experiments by Ogawa):

The possibility that methemoglobin arises simultaneously during the process of choleglobin formation in the system of Hb—ascorbic acid —O_2 was suggested by Lemberg (*1*) as early as 1941. Lemberg takes the view that methemoglobin enters the cycle of choleglobin formation. The simultaneous formation of met Hb in this system was finally proved by Kikuchi (*9*), who could recognize the production of CN-met Hb in the reaction system of choleglobin formation by the addition of KCN to the system (*cf.* also Holden (*10*)). Met Hb can not, however, be an intermediate of choleglobin formation in this reaction process, since choleglobin will not be formed from methemoglobin by the direct action of H_2O_2. It should first be reduced to hemoglobin to be able to enter the cycle of choleglobin formation. Methemoglobin can undoubtedly be reduced to hemoglobin by ascorbate (CN-met Hb, however, can not). Thus, if methemoglobin, instead of hemoglobin, was used as the starting substance in the choleglobin forming system, the formation of choleglobin will start after the reduction of methemoglobin to hemoglobin. Experiments on this subject was already reported by Lemberg (*1*) and Takeya (*4*), but not yet fully studied. Thus we have now reinvestigated their studies.

The results of our experiments of methemoglobin without catalase are given in Fig. 9 and that of methemoglobin containing catalase in Fig. 10. The time curve of choleheme production in both experiment

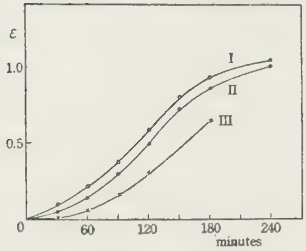


FIG. 9. Formation of choleheme from metHb containing no catalase.

Reaction mixture: 2.0 ml. 0.6% metHb, 1.6 ml. *M*/15 phosphate buffer (pH 7.38) and 0.4 ml. ascorbic acid. The final concentration of the latter: I, 1.76; II, III, 0.88 mg./ml. Temperature: I, II, 37.5°; III, 31–32°.

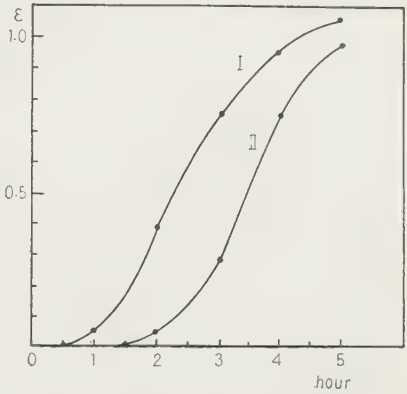


Fig. 10. Formation of choleheme from metHb containing catalase.

Reaction mixture: 5.0 ml. 0.6% metHb, 4.0 ml. *M*/15 phosphate buffer (pH 7.38) and 1.0 ml. ascorbic acid. The final concentration of the latter: I, 1.76; II, 0.88 mg./ml. Temperature: 37.5°

series was significantly S-shaped, especially in the lower ascorbate concentrations. This is more striking in the presence of catalase. The final amount of choleheme production in these experiments was found, however, to be almost the same as that in the experiments with hemoglobin (Fe^{++}) (see also Takeya). And the reaction velocity of choleglobin formation after the reaction started from the apparent lag phases in these experiments does not differ much from that in hemoglobin (Fe^{++}) experiments. The velocity curves of both series of experiments are in great similarity. In the oxidation of ascorbate, however, the typical S-shaped reaction curve is not formed, since its decomposition is fairly vigorous already in the initial stage. This is surely due to the greater oxidation of ascorbate used for the reduction of methemoglobin to hemoglobin.

V. Temperature Effect (Experiments by Ogawa):

The temperature effect on the present reaction process is singificantly great. With the decrease of temperature, the velocity curves both for the choleheme formation and for the ascorbate oxidation (both in the presence or absence of catalase) are more S-shaped, and the increase of the length of lag phase is marked, especially in choleheme formation, as shown in Fig. 11 and 12. The greatest temperature effect

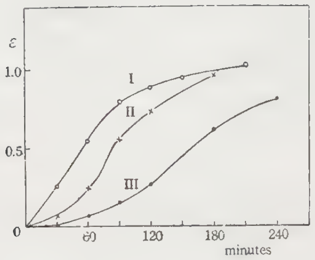


FIG. 11. Effect of temperature on the reaction of choleglobin formation (reaction without catalase).

Final concentrations of ascorbic acid: 0.88 mg./ml. Temperature: I, 32.5°; II, 31–32°; and III, 26–27°.

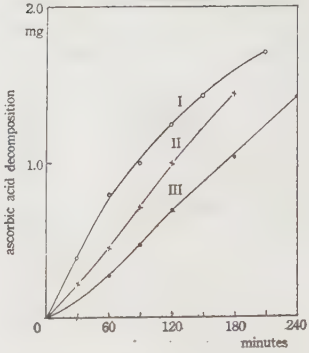


FIG. 12. Effect of temperature on the decomposition of ascorbic acid.

Reaction condition: same as described in Fig. 11.

was observed in the reaction with methemoglobin containing catalase.

VI. The Effect of pH (Experiments by Ogawa):

The experiments were carried out in a variety of pH from 4.8 to 11.37. The following buffer mixtures were used:

pH 4.8 . . . . acetate buffer,
pH 5.9-8.04 . . . . . phosphate buffer,
pH 9.37-11.37 . . . . Na_2CO_3-$NaHCO_3$ buffer.

The experiments were carried out with hemoglobin solution obtained from normal human blood containing catalase (Fig. 13) and those from non-catalase human blood (Fig. 14). The initial rate of the green pigment formation, in both series of the experiments, becomes greater as the pH increases. In the later stadium, however, the highest green pigment formation was obtained at the neutral pH range. Similar results were obtained in experiments with methemoglobin. Generally speaking, pH 6 to 7 seems to be the optimum condition for the reaction of choleheme formation. Above the pH 9, some different product other than choleglobin seems to have been formed (the suggested green pigment seems to be able to liberate its iron with great difficulty—Yamada, see below).

In the above-mentioned experiment series, the oxidation of ascorbate was followed up. The rates of the oxidation is accelerated simply in proportion to the increase of pH value.

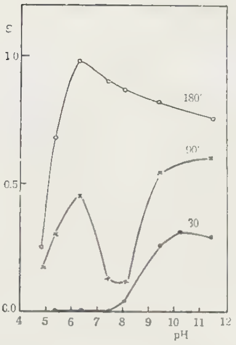


FIG. 13. Effect of pH on the decomposition of hemoglobin (reaction in the presence of catalase).

Reaction mixture: 2.0 ml. 0.6% O_2Hb, 1.6 ml. buffer solution and 0.4 ml. 8.8 mg./ml. ascorbic acid. Temperature: 37.5°.

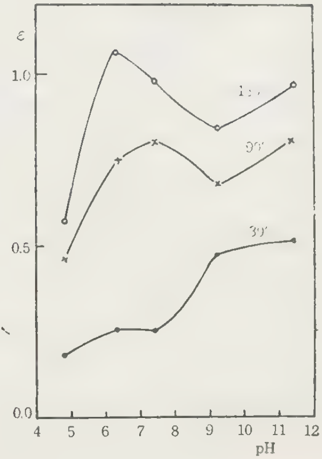


FIG. 14. Effect of pH on the decomposition of hemoglobin (reaction without the presence of catalase).

Reaction mixture and the temperature: same as described in Fig. 13.

VII. The Splitting of Iron from Choleglobin (Experiments by Yamada):

It is already known that choleglobin can split its Fe easily when treated with 0.4 per cent HCl. This is assumed by many to correspond to the "easily splittable iron" of Barkan (*5*). In the present study we have quantitatively followed up the amounts of these easily splittable iron arising in the reaction system during choleglobin formation.

By the experiments with a variety of ascorbate concentrations, it was shown that, as given in Fig. 15, the amount of the splitted Fe was found to agree with that calculated from the amount of choleheme formed in the experiments. Fairly good agreement was seen in the more early stadium of the reaction. In the later stadium, it was found that the amount of splittable iron determined was greater than that calculated from the choleheme formed. This seems to be due to the further decomposition of choleheme occurring by longer duration which leads possibly to some compound with lower optical density in the determined wavelength region.

The calculation of Fe amount corresponding to that of choleheme

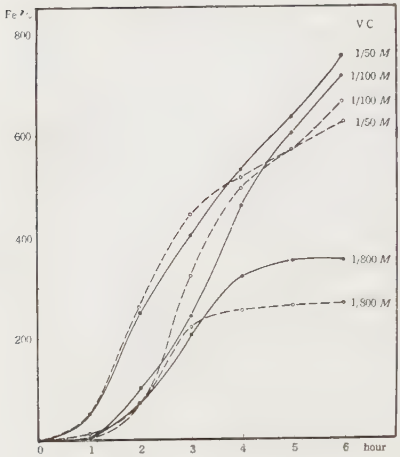


FIG. 15. Detachment of iron from choleglobin (No. 1).

0.3% Hb, pH 7.38, and temperature, 38°. Full line: iron found. Dotted line: iron calculated from the amount of choleheme found.

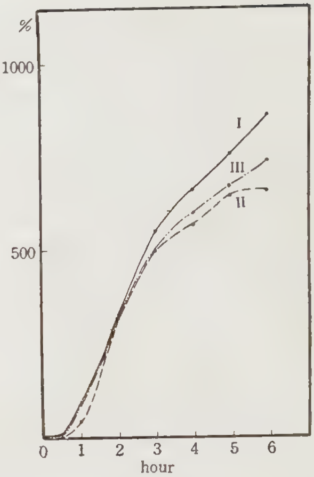


FIG. 16. Detachment of iron from choleglobin (No. 2). 0.3% Hb with $M/100$ ascorbic acid, pH 7.38, and temperature, 38°.

I, splitted iron found after the treatment with 0.4% HCl.

II, iron claculated from the amount of choleheme formed.

III, splitted iron found after the treatment with trichloracetic acid only.

found was made as follows: since $\varepsilon_{\text{mol.}}$ for choleheme $= 1.36 \times 10^4$ as described above and choleheme molecule contains 1 Fe atom (at.wt. $= 55.84$), so in the concentration of choleheme of $1 \times 10^{-4}\ M$ and $\varepsilon_{618} = 1.36$, for example, the amount of Fe in the 100 ml. of choleheme solution can be calculated as $55.84 \times 10^{-4} \times 10^{-1} = 558.4\ \gamma$.

Now, the reaction solution was treated with 0.4 per cent HCl solution and deproteinized by addition of trichloracetic acid. The resulted precipitate was dissolved in NaOH solution and the absorption property was studied spectrophotometrically after $Na_2S_2O_4$ was added to the solution. The absorption in the green region corresponded to those of alkali denatured hemochrome and in the red, only a diffuse absorption. It will be then assumed that, there remains no appreciable amount of choleheme in the solution, and that the liberation of Fe from choleheme is thus completed.

Now, the determination of splittable Fe was carried out with the reaction solution which is treated with trichloracetic acid alone without preliminary treatment of 0.4 per cent HCl. The Fe value thus determined as shown in Fig. 16, was found to be almost the same as that treated with HCl. Furthermore the Fe values determined by this simple procedure corresponds more accurately to the choleheme value than that determined by the former procedure. By the designation E.S.I. has been meant that part of Fe, which is splittable from some of the specific porphyrin Fe by being treated with 0.4 per cent HCl. If, however, this Fe can be detachable by trichloracetic acid as easily as by the treatment with HCl, the definition of E.S.I. should now be altered in some points. We are not yet in the situation to have any explanation whether the discrepancy of the Fe values obtained by both procedures is related to the chemical difference of the reaction products in the given system.

DISCUSSION

One of the remarkable facts observed through the present experiment series is that the reaction curves of both ascorbate oxidation and choleheme formation showed a similar S-shaped type. And, in general, the rises of the decomposition curve of ascorbate precede those of choleheme formation. The inclination of the curve to the S-shape is more remarkable in the lower ascorbate concentrations and especially in the presence of catalase. In the absence of catalase (*cf.* Lemberg (*11*)) and in the higher ascorbate concentrations no S-shaped curve is formed (Fig. 4). This seems to be caused by the shortning of the lag phase. Also in the presence of catalase, when the ascorbate concentrations are kept higher over certain limit, the difference of the rates of choleheme formation due to the difference of ascorbate concentrations mainly results from the length of the lag phase. After the lag phase shifted to log phase, the difference of the rates of choleheme formations can be seen no more; it may be accepted that the chemical process in this stadium is sufficiently active to overcome the inhibitory effect of catalase, although the effect from the possible inactivation of catalase during the reaction process can hardly be overlooked.

From all these facts, the chemical process of the present reaction can be assumed to be of the nature of chain reaction: H_2O_2 is produced by the oxidation of ascorbate, choleglobin is then formed from hemoglobin by the action of the former and the oxidation of ascorbate is now strongly promoted along with the process of the latter reaction. This chain reaction nature of choleheme process may, however, not be solely attributed to the determining reaction of ascorbate oxidation. The facts observed in the following experimental series dealing with the oxidation rate of ascorbate, as shown in Fig. 17 may be suggestive for the explanation of the reaction nature as a whole. Curve I in Fig. 17 shows the ascorbate oxidation in the phosphate buffer only, II shows that with eggalbumin added and III shows that with hemoglobin added in the same protein concentration as in II. As shown in the figure, the reaction I and II proceed both straight lineal and the autoxidation of ascorbate is strongly depressed, however, in the system II. In the system III, however, the initial rate of ascorbate oxidation is nearly the same as that of II, while after a certain space of time the oxidation rises promptly to its rate, so that a distinct S-shaped curve is formed. For this, some sort of hemoprotein possibly arising in the system during the process must probably be responsible. In other words, it may be suggested that some of the intermediates or products during the process of forming choleglobin from hemoglobin posesses an especially high activity of catalysis in the oxidation of ascorbate.

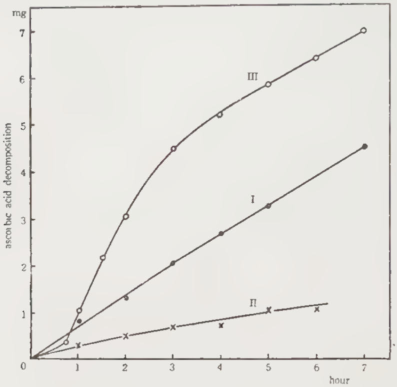


FIG. 17. Difference in the rate of ascorbic acid decomposition, in the presence and in the absence of hemoglobin.

I, 5.0 ml. redestilled water, 4.0 ml. $M/15$ phophate buffer (pH 7.38) and 1.0 ml. 17.6 mg./ml. ascorbic acid (neutralized).

II, 0.6% egg albumin solution was mixed in place of water in I.

III, 0.6% O_2Hb solution was mixed in place of water in I. Temperature, 37.5°.

For the possible hemeproteins under consideration, methemoglobin, choleglobin and cholehemochromogen may first be mentioned. In the following experiments, therefore, the catalytic activity of these compounds were studied.

Methemoglobin—This is more evidently indicated in Fig. 18 and 19. These figures show the reaction rate curve of the process with O_2Hb and with methemoglobin. The rates of choleheme production and ascorbate oxidation run almost completely parallel. From this, it is concluded that the catalytic activity of methemoglobin is not so strong as to explain the total facts.

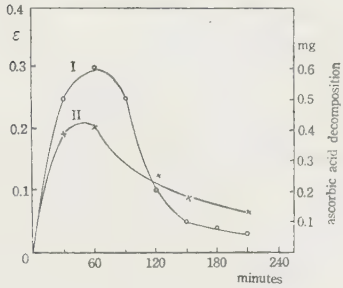


FIG. 18. Relation between the rates of choleheme formation and of ascorbate decomposition. Part 1: Starting from O_2Hb without catalase.

Mixture: 2.0 ml. 0.6% O_2Hb, 1.6 ml. $M/15$ phosphate buffer (pH 7.38), and 0.4 ml. 17.6 mg./ml. ascorbic acid. Temperature, 37.5°.

I, choleheme formation, II, ascorbic acid decomposition.

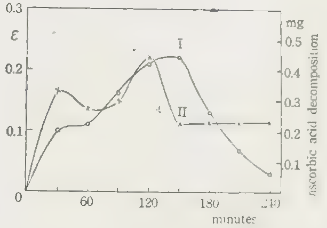


FIG. 19. Relation between the rates of choleheme formation and of ascorbic acid decomposition. Part 2: Starting from metHb without catalase. Reaction condition: same as described in Fig. 18.

I, choleheme formation. II, ascorbic acid decomposition.

Choleglobin and Cholehemochromogen—The production of choleheme was studied under the same conditions as in the experiment in Fig. 2 and in three fractions separately; (I) with whole reaction solution (choleglobin+cholehemochromogen), (II) with supernatant solution (choleglobin) and (III) with the precipitate (cholehemochromogen). The results are shown in Fig. 20. The curve of cholehemochromogen formation (III) runs by no means parallel with those in (I) and (II), the peak of the former curve appears significantly delayed compared with those of the latter. The " choleheme formation " means thus the " choleglobin formation "; the formation of choleheme begins, at least, from the solute state of hemoglobin.

In consideration of above facts and studying the curves in Fig. 18 and 19, it will be assumed that the rate of ascorbate oxidation is in parallel with that of choleglobin formation (ascorbic acid is oxidized along with the choleglobin formation) and that the cholehemochromogen may have a slightest, if any, catalytic activity in the oxidation of ascorbate.

Further, as already mentioned in the Experiment *I*, the content of soluble choleglobin in the supernatant solution remains only in limited extent. After 3 hours, the content of choleglobin formed remains almost in constant. The rate of oxidation of ascorbate, however, reaches to its maximum in about 3 hours and it decreases generally after that. This indicates that the catalytic activity of choleglobin in the ascorbate oxidation is also insignificant. Thus, though it is clearly demonstrated that the ascorbate is oxidized in the highest degree just during the process of choleglobin formation, we are not yet in a position to point out any compound in the present system which is responsible for such an energetic catalytic activity. It is highly probable that some sort of hemeprotein may be formed in the system, which is so far unknown, but which posesses much more catalytic activity for ascorbate oxidation than any of the known compounds existing in the system. It is further suggested that such a hemeprotein may be formed as an active intermediate of choleglobin from hemoglobin.

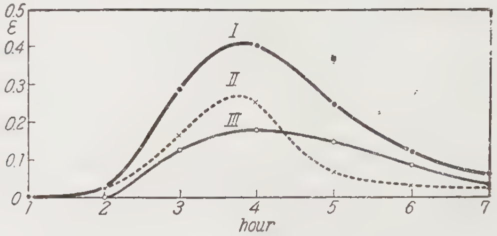


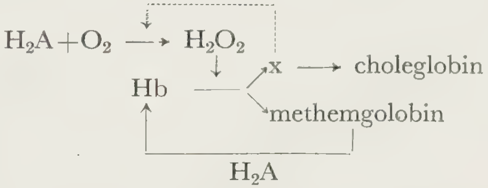
FIG. 20. Changes in the rate of choleheme formation in the course of the reaction. The rates are expressed in terms of the amount of y component formed in the reaction solution in each interval of time. Amount of y was obtained from ε_{618} of the reaction solution after treatment with $Na_2S_2O_4$ and NaOH.

I, reaction solution as a whole (choleglobin+cholehemochromogen)
II, supernatant (choleglobin)
III, precipitates (cholehemochromogen)

The effect of temperature upon the process of choleglobin formation is much more significant than that on ascorbate oxidation. This suggests that the formation of choleglobin is by no means controlled merely by the amount of H_2O_2 formed from ascorbate. It may further be presumed that for the initiation of the process of choleglobin formation from hemoglobin, a slightest grade of denaturation, which is reversible and in the meaning of Holden's "perturbation" in the molecule of the latter, may have a decisive role. And the "active intermediate" suggested above can be one of the hemeproteins of such a modification.

The yield of choleheme of the process in the system of Hb-ascorbate-O_2 is by no means theoretical; there remains always 20-25 percent of unaffected protoheme in the system. And if the globin is previously denatured by NaOH, choleheme can not be formed even by direct addition of H_2O_2. Taking these facts into consideration, it can be assumed that the protein part of hemoglobin should be in its native state, at least in limitted extent, to start with the formation of choleglobin. The relationship between the disintegration of heme part and the property of globin part of the hemoglobin molecule seems to be of great interest for the chemical mechanism in question.

Finally, on the basis of our experimental findings and of our assumption discussed above, a hypothetical reaction scheme for the process of choleglobin formation will be postulated as follows:



in which H_2A means ascorbic acid. Through the action of H_2O_2, formed from ascorbate by oxidation with O_2, hemoglobin undergoes autoxidation leading to the formation of methemoglobin on one hand and of an unknown active intermediate x on the other. This x will further be converted into choleglobin. By the catalytic action of compound x, highly active when once formed, the production of H_2O_2 will be accelerated and this will lead to the increasing formation of x and choleglobin. The process forms thus a reaction cycle and is accelerated by the increase of x. The chain reaction nature of the present reaction as a whole seems to be attributed to the existence of a hypothetical intermediate x which is strongly catalytic.

SUMMARY

A variety of experiments were carried out in order to analyze the chemical mechanism of choleglobin formation in the system of hemoglobin, ascorbic acid and oxygen. The results of the experiments are summarized as follows:

1. By treating the reaction solution with NaOH and $Na_2S_2O_4$,

the choleheme protein is converted to alkali denatured cholehemochrome which can be determined by means of spectrophotometry. The method enables us to follow up the present reaction process quantitatively and with fair exactness.

2. From the optical density of alkali denatured cholehemochrome at the wavelength of 618 mμ as experimentally determined, $\varepsilon_{\text{mol.}}$ for choleheme is calculated as 1.36×10^4.

3. The structural change which occurred in the heme part of choleglobin is just the same as in that of cholehemochromogen.

4. During the process of choleheme formation in the reaction system without catalase, almost 20 moles of ascorbic acid are used up per mole of protoheme.

5. The reaction curves of choleheme production and ascorbate oxidation are in parallelism. Both of the curves are characteristic in their S-shaped type. The slope of the S-shape is pronounced either with the decrease of ascorbate concentration or by the presence of catalase or further with the lowering of the temperature.

6. Choleglobin can be obtained from hemoglobin by the direct action of H_2O_2 upon the latter and without O_2. This reaction can proceed whether ascorbate is present or not so far as the hemoglobin is kept in its Fe^{++} state. Methemoglobin (Fe^{+++}), however, can not produce choleglobin by the same oxidant.

7. In the system of methemoglobin, ascorbic acid and O_2, methemoglobin must be first reduced to hemoglobin in order to be oxidized to choleglobin.

8. Choleglobin seems to be able to liberate iron completely by the treatment with 0.4 per cent HCl as well as by the treatment with trichloracetic acid in the deproteinization procedure.

9. Denaturation of globin part have, most likely, some important influence upon the disintegration of heme part.

From the above results, it may be concluded as follows: choleglobin is formed from hemoglobin by the action of H_2O_2 produced from ascorbic acid and O_2 in which the heme molecule itself serves as a peroxidative substrate for its own molecule. It is further suggested that in this process some sort of intermediate interposes, which must be highly active as an oxidative catalyst. Finally, a hypothetical scheme of the reaction process of choleglobin formation is presented by assuming an active role of this probable intermediate.

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BIOSYNTHESIS OF VITAMIN B_{12} IN VARIOUS ORGANISMS. I

By YOSHIKAZU SAHASHI, KIICHI IWAMOTO, MASAYUKI MIKATA, AKIHIKO NAKAYAMA, HEIICHI SAKAI, JUSAKU TAKAHASHI, JUNZO HAYASHI, NOBUO SENO, TADAMI AKATSUKA, TAIHEI MIKI, KEIJI HARASHIMA AND RENTARO MATSUMOTO

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(Received for publication, February 20, 1953)

INTRODUCTION

Recent studies on the biochemistry of vitamin B_{12} are worthy of deep interest and numerous papers have been published on the subject, dealing with chemical, physiological and applicational aspects, but the mechanism of biosynthesis of this vitamin has not been investigated so far. The authors carried out the present research, keenly feeling the necessity of it, especially in a country like Japan poor in animal protein factor resources.

EXPERIMENT 1. CATTLE

During 1950–52, the authors (Sahashi and Iwamoto) carried out several experiments with vitamin B_{12}-deficient cattle in Shiga Agricultural College. At first, the authors attempted to study the etiology of the endemic disease in cattle known as " Kuwazu disease " in the province from the view-point of vitamin B_{12}-forming metabolism. From the eariest time, this endemic disease in cattle has been studied by numerous investigators, but few notable reports have appeared on this problem.

Detective Experiments of Vitamin B_{12}-forming Actinomycetes in Feces of Diseased Cattle (1, 2)—In 1948, R. T. Lillie obtained vitamin B_{12} active substances from cow manure. In 1949, Sakai isolated a few active *Streptomyces* from the pasture in Chiba and Hokkaido in Japan, and in 1950 a new strain of *Actinomycetes* (C. R. 21, Sakai) from ordinary cow rumen.

Guided by the above literature, the present authors at first carried out detective researches of vitamin B_{12}-forming microorganisms from feces of the endemic " Kuwazu disease."

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Concerning the studies of the cultural detection of *Actinomycetes* in feces of cattle, no paper has been published.

Several intensive cultural experiments of the microbe by the Czapeck medium were repeated, and in feces of 18 cattles suffering from the "Kuwazu disease," the presence of *Actinomycetes* was not proved (*1*, *2*). On the other hand, control tests for ordinary animals always showed positive results. The study on the absence of this microorganism was therefore taken up as a part of the above research project.

Toxic Effect of SiF_6^{--} *Ions on the Growth of Actinomycetes* (*3*)—After repeating the analysis of water in the district, SiF_6^{--} ions were found, and its toxic power on the growth of the microorganism was studied. It was found that 0.8~1.0 mg. fluorine (as SiF_6^{--} ions) per 10 ml. of the culture medium for microbial assay was fatally toxic to *Actinomycetes*. The toxicity of fluorine ion (F^-) was rather week towards this microbe.

Moever, biosynthetic ability of vitamin B_{12} by *Actinomycetes* isolated from the farm-soil of Hira-district was found to be extremely low. The toxic effect of the above fluorine compound on the biosynthesis of vitamin B_{12} by *Actinomycetes* was accurately tested with Merck's Na_2SiF_6, and its strong inhibitive action on the growth of the microbe was affirmatively determined. The synthetic activities of vitamin B_{12} by ordinary *Actinomycetes* were also affected by toxicity of SiF_6^{--} ions, even if in the cobalt sufficient medium (Table I).

TABLE I

Synthetic Activity of Vitamin B_{12} *by Actinomycetes in the Presence of* SiF_6^{--} *and* Co^{++}

Co content in 10 ml.	F content in 10 ml.	Growth of *Actinomycetes*	Vitamin B_{12} activity
1. 0.015 *mg.*	0.0 *mg.*	+++	100 *per cent*
2. "	0.1	+++	100
3. "	0.2	+++	100
4. "	0.3	+++	80
5. "	0.5	++	64
6. "	0.6	±	52
7. "	0.8	−	0
8. "	1.0	−	0

F . . . . Merck's Na_2SiF_6 (as F), Co . . . . Kahlbaum's $CoSO_4.7H_2O$ (as Co), Czapeck medium, 37°, 68 hours.

The Appearance of "Kuwazu disease" in Ordinary Cattle Treated with the Water Containing SiF_6^{--} Ions (4, 5)—Feeding experiments with 3 normal calves were carried out in the same condition as the diseased animals in the Hira-district. At the biggining of the experiments, the presence of *Actinomycetes* in feces was previously detected and the experiments were continued for ten months. After 30 days' feeding, *Actinomycetes* was negative in the excrement.

After 10 months' feeding, the animals indicated the typical symptoms and red blood cell counts were quite similar to those of the ordinary "Kuwazu disease" (Table II, Figs. 1 and 2).

TABLE II

Variation of Red Blood Cell Counts during the Experiment

Oct. 1951	$800\times10^4/mm^3$ (at the biginning of the Experiment.)			
Nov. ,,	$900\times10^4/mm.^3$	Jun.	1952	$913\times10^4/mm.^3$
Dec. ,,	760 ,,	Jul.	,,	585 ,,
Jan. 1952	621 ,,	Aug. 11,	,,	576 ,,
Feb. ,,	791 ,,	,, 26,	,,	480 ,,
Mar. ,,	——	Sep. 5,	,,	430 ,,
Apr. ,,	750 ,,	Oct. 3,	,,	440 ,,

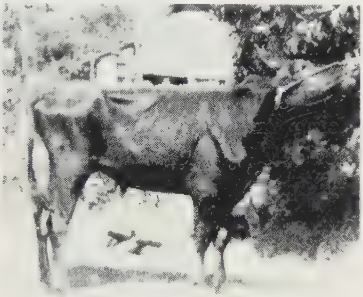


FIG. 1. Cattle suffering from "Kuwazu Disease"

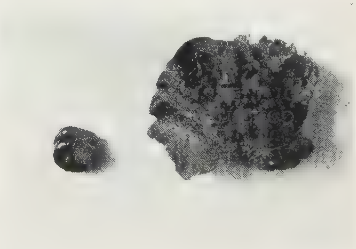


FIG. 2. Feces of the diseased cattle (left). Feces of ordinary healthy cattle (right)

Curative Experiments of Vitamin B$_{12}$-deficient Disease (4, 5)—The diseased animals thus obtained were treated by the intramuscular injection of 30 γ crystalline vitamin B$_{12}$ in aqueous solution and the satisfactory response on the curative effects were observed. The variation of red blood cell counts and of the diameter of red corpuscles are shown in Table III and Fig. 3.

TABLE III

Response of Erythrocyte to Injection of Vitamin B_{12}

Experiment 1	Just before injection	$480\times10^4/mm.^3$	Experiment 2	Just before injection	$435\times10^4/mm.^3$
	3 hrs. after "	476 "		27 hrs. after "	476 "
	16 " " "	535 "		30 " " "	650 "
	51 " " "	720 "		36 " " "	801 "
	65 " " "	870 "		44 " " "	643 "
	10 days " "	425 "		6 days " "	590 "
				10 days " "	440 "

Further Experiments for the Detection of Other Strains with Vitamin B_{12}-producing Activity—Finally, the authors carried out further studies on several kinds of strains in rice-fodder such as Natto bacilli belonging to *Bacil. subtilis* (or *Bacil. mesentericus* group) but these microbes differed from *Actinomycetes* in the vitamin B_{12}-producing activity.

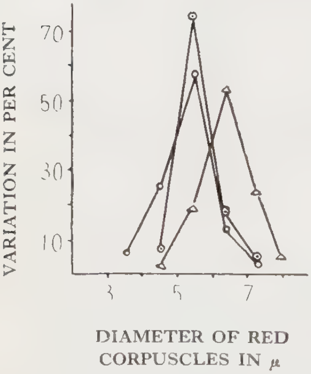


FIG. 3. Variation of the red blood cell counts and of the diameter of corpuscles.
Before injection, —△—△—
After injection, —○—○—
Normal, —◉—◉—

From the above findings, the present authors have concluded that "Kuwazu disease" in Shiga Prefecture in Japan may be a form of macrocytic anemia, namely endemic vitamin B_{12} deficiency in cattle owing to the absence of vitamin B_{12}-producing microorganisms due to the toxic power of SiF_6^{--} ions.

The Absence of Vitamin B_{12}-forming Actinomycetes in Cattle Suffering from "Kuiyami Disease" (*6, 7*)—In the mean while, the present authors carried out a few microbiological studies on feces of the cows suffering from "Kuiyami disease" in the Co-deficient zone of Hyogo Prefecture in Japan. In this case also, no *Actinomycetes* was detected in feces. This result seemed to be similar to the Co-deficiency in animals reported in a recent paper by Becker. (*8*) However that may be, the absence of *Actinomycetes* in "Kuiyami" feces must be an important finding for the clinical test of disease in cattle.

The Absence of Vitamin B_{12}-forming Actinomycetes in "Kiriyoi" Feces (9)—More recently, the above researches were noticed by N. Seno who has been studying "Kiriyoi disease" of cattle in Shimane Prefecture in Japan from his veterinary scientific view-points since a few years ago. During his clinical researches, N. Seno recognized severe anemia in the symptoms of "Kiriyoi disease" besides vitamin D deficiency.

Then, observations of crucial importance were made by the present authors with the cultural tests of *Actinomycetes* in the feces of "Kiriyoi" diseased cattle. All experiments failed to find the presence of vitamin B_{12}-producing microorganisms in feces of the diseased animals, as in the case of "Kuwazu disease." On the contrary, control experiments for healthy animals in the province always indicated positive results. Moreover, the isolation of *Actinomycetes* from the diseased cow-rumen by Czapeck medium was also negative. On the other hand, further studies on the vitamin B_{12}-producing activities with the other strains isolated from the above cow-rumen were made. The content of vitamin B_{12} in broth was estimated by Euglena assay, but all were negative.

The Curative Experiments of "Kiriyoi Disease" with Vitamin B_{12} (9)—A curative experiment of "Kiriyoi disease" in cattle was carried out in the same way as shown in case of the "Kuwazu disease" by the intramuscular injection of crystalline vitamin B_{12} solution. After treated with 10 γ crystalline vitamin B_{12} in aqueous solution by injection two times, red blood cell count increased from 488×10^4 mm^3 to $640 \times 10^4/\overline{\text{mm}}^3$, and subsequently, all the symptoms of the "Kiriyoi disease" completely disappeared. On the other hand, the authors succeeded also in a few curative treatments of severe symptom with administration of 30 γ vitamin B_{12} besides vitamin D by intramuscular injection.

The Prophylactic Tests of "Kiriyoi Disease" with Vitamin B_{12} (9)—In the mean while, one of the authors (N. Seno) carried out repeated prophylactic experiments with 70 calves. The calves were divided into two groups: 53 calves were treated with vitamins B_{12} and D, and 17

TABLE IV

Prophylactic Tests on "Kiriyoi Disease"

	Number of diseased animals at the end of experiment
Experimental group	2/53
Control group	6/17

calves were kept as control without the treatment, with the results shown in Table IV.

From these findings, " Kiriyoi disease " was also considered to belong to the same kind of vitamin B_{12} deficiency owing to the absence of vitamin B_{12}-producing *Actinomycetes.*

EXPERIMENT 2. RATS

Recently, the authors (Nakayama and Matsumoto) have attempted to study a biochemical chain-relation between vitamin B_{12} and phosphate metabolic system in bone by the effect of vitamin B_{12} upon the value of vitamin D determined biologically, and found a fact similar to that of the biosynthetic ability of vitamin B_{12} in rats fed on this vitamin-deficient diet.

In 1950, R. W. Vilter *et al.* suggested a probable schematic representation of a chemical-chain-relation between nucleic acid and vitamin B_{12} in vivo. In 1951, at Nagoya University, S. Ito observed the simultaneous increase of both ATP and red blood cell counts in bone marrow test.

From these interesting communications, and from the fact that vitamin D is an important factor in the phosphorus metabolism, the authors attempted to study a biochemical chain-relation between vitamin B_{12} and phosphate metabolic system.

Then, experimental procedure and results are as follows:

Effect of Vitamin B_{12} upon the Value of Vitamin D with Rats Fed on Sterol-free and Vitamin B_{12}-containing Diets—Based on the experimental evidence that the sterols may be eventually proved to be the mother substance of vitamin D, and that when cholesterol tagged with deuterium is fed to women, pregnandiol containing the isotope could be recovered from the urine, the authors used the synthetic diet completely freed from sterols and accurate results for the bioassay of vitamin D were obtained (*10*). The composition of the diet containing animal protein (purified horse meat meal) is shown in Talbe V.

The content of vitamin B_{12} in purified horse meat meal was estimated by Euglena assay after digestion with pancreatin. Ordinary Steenbock's diet from vegetable source, however, contained no vitamin B_{12} when tested in the same manner.

Effect of Vitamin B_{12} on the Production of Rachitis in Rats (11)—The experiments were carried out with ordinary Steenbock's diet composed of a mixture of American yellow corn meal 76, dried wheat gluten meal 20, $CaCO_3$ 3, and NaCl 1.

TABLE V

Rachitogenic Diet Containing Vitamin B_{12}

Constituents		Composition	Content of the each constituent					Lieberman's reaction & trichloroacetic acid reaction of sterols
			Ca	P	Vitamin B_1	Vitamin B_2	Vitamin B_{12}	
		per cent	*per cent*	*per cent*	$\gamma/100g$	$\gamma/100g$	$\gamma/100g$	
Purified rice powder		83	0.009	0.087				—
Purified horse meat meal		11	0.051	0.119			$5\sim7$	—
Salts	$CaCO_3$	3	40					—
	NaCl	1						—
Dry yeast extracted with ether		2	0.024	1.530	5,500	5,000		—
β-Carotenes (Ethyl oleate solution)		1 drop per rat daily, containing $6\sim20\,\gamma$ of β-carotene ($\fallingdotseq$10 I.U.$\sim$30 I.U. of vitamin A)						

The animals were divided into two groups. The 1st group was administered with 3 γ of crystalline vitamin B_{12} in aqueous solution per rat every 5 days through intramuscular injection, while the 2nd group served as the negative control. There was found no difference between the two groups in the estimation of the healing degree of rachitis by the x-ray photography after the prophylactic period of one month (Table VI).

Effect of Vitamin B_{12} on Curative Test of Ricket in Rats with Vitamin D—Vitamin D-deficient animals treated as above mentioned were kept under the additional oral feeding of 3 γ of vitamin B_{12} every 5 days and 1 I. U. of vitamin D_2 daily.

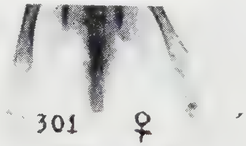
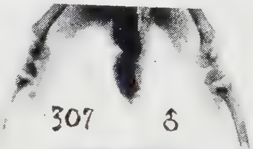
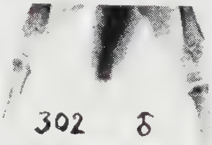
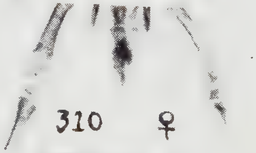
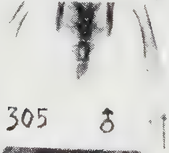
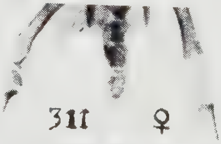
No effect of vitamin B_{12} was exerted in the curative period of six days (Table VII).

Relation of Red Blood Cell Counts and Serum Phosphate to the Degree of Ricket in Prophylactic Period—The variation of number of red corpuscles and phosphorous content in serum were exactly tested. No significant difference was seen in both experimental groups (Table VIII).

TABLE VI

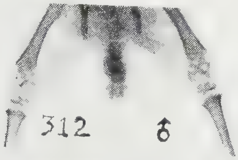
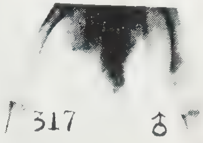
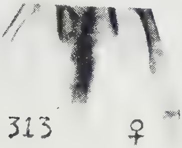
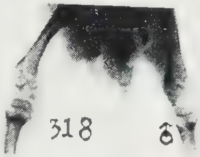
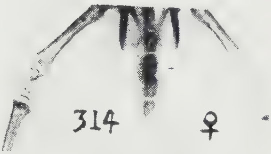
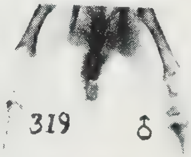
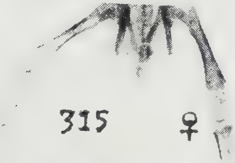
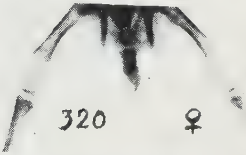
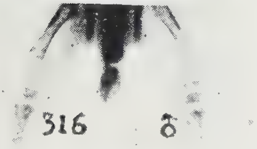
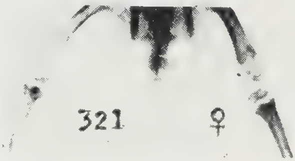
Influence of Vitamin B_{12} on Bourdillon's Scale for Prophylactic Tests

Control Group

Bourdillon's scale		Bourdillon's scale	
1		1	
0		0	
1		2	
2		2	
1		2	
0		0	

mean 1.0

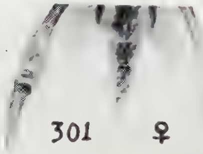
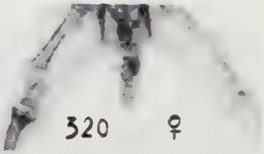
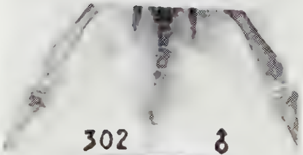
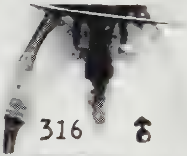
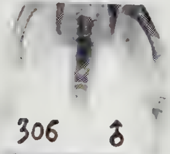
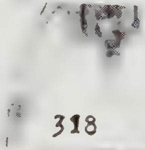
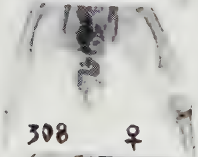
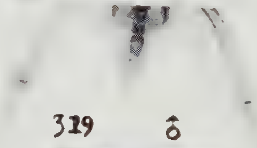
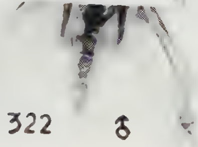
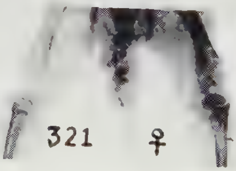
Test Group
(V. B_{12} 3 γ/5 days)

Bourdillon's scale		Bourdillon's scale	
2	 312 ♂	2	 317 ♂
1	 313 ♀	0	 318 ♂
2	 314 ♀	0	 319 ♂
2	 315 ♀	0	 320 ♀
0	 316 ♂	0	 321 ♀

mean 0.9

TABLE VII

Influence of Vitamin B_{12} on Bourdillon's Scale for Curative Tests

	Control Group Bourdillon's scale	Control Group	Test Group (V. B_{12} 3 γ/5 days) Bourdillon's scale	Test Group (V. B_{12} 3 γ/5 days)
Without V. D_2	1	 301 ♀	0	 320 ♀
With V. D_2	6	 302 ♂	3	 316 ♂
	4	 306 ♂	6	 318 ♂
	5	 308 ♀	5	 329 ♂
	7	 322 ♂	5	 321 ♀
	mean 5.4		mean 4.8	

From the above findings, complete nutrition in rats fed on vitamin B_{12}-deficient diet seems to be due to the biosynthesis of this vitamin.

EXPERIMENT 3. BIRDS

It is well known that many investigations on the requirement of

TABLE VIII

Relation among Degree of Ricket in Rats, Red Blood Cell and Serum Phosphorous

	Rat No.	Degree of healing in prophylactic period	Number of corpuscles in blood	P in serum
			million/mm³	*mg. per cent*
Control group	301	1	6.46	
	302	0	6.37	7
	303	1	8.31	6.5
	305	1		4.7
	306	0	8.67	
	308	0	6.76	5.1
	310	2		5.6
	322	0	7.25	5.6
Test group (receiving V. B_{12})	312	2	7.20	4.7
	315	2		5.1
	316	0	8.13	4.7
	317	2		7.5
	318	0	8.22	
	319	0	9.21	5.4
	320	0	8.45	4.2
	321	0	7.86	4.2

vitamin B_{12} to young chicks have already been made and the supply of vitamin B_{12} resulted in their excellent response in growth and hatchability. But, on the other hand, many workers previously obtained vitamin B_{12} active substances from poultry-farm, and the present authors (Sakai *et al.*), in Japan (1950), also isolated a few kinds of strong strains (PD-61 Sakai) producing vitamin B_{12} (13500LLD units/ml. broth) from poultry-farm. Recently, the authors (Sahashi, Iwamoto and Sakai) have attempted to study a relation between the vitamin B_{12} nutrition in poultry and vitamin B_{12} producing power of *Actinomycetes*.

Growth Experiments in Poultry with fresh Actinomycetes Supplement (*12*)—50 young chicks were previously fed on vitamin B_{12} deficient diet, composed of a mixture of corn meal 100, wheat bran 55, rice bran 80, rice embryo 100, wheat flour 30, Italian millet 80. After 11 days, chicks were divided into two groups. The 1st group of 25 chicks weighing 16 g. each were orally administrated with a minute quanitity of fresh *Actinomycetes* supplement every other day, while the 2nd group of 25 chicks served as the negative control. No satisfactory response was seen in the above experimental data, although a slight increase in body weight of the 1st group chicks resulted more than in control group.

Detective Experiments of Actinomycetes in Chick's Alimentary Canal administered with Fresh Actinomycetes (*13*)—Several detective isolations of vitamin B_{12}-producing micro-organisms belonging to *Actinomycetes* were repeated using Czapeck medium from chick's alimentary canal, but no microbe was found in proventriculus nor intestines, even when administered with fresh *Actinomycetes.*

Failure of Isolation of Actinomycetes in Normal Healthy Poultry (*13*)—Further detective experiments of vitamin B_{12}-forming microbes were continued in 11 ordinary healthy poultry. All results were completely negative (Table IX).

TABLE IX

Detective Failure of Actinomycetes

Organs	Crop	Stomach	Jejunum	Intestine	Caecum
Presence of *Actinomycetes*	0	0	0	0	0

Moreover, the isolation *Actinomycetes* from rectum or cloaca of 5 popultries (♂ 3, ♀ 2) were also all completely negative.

The Absence of Actinomycetes in Feces (*Freshly Excreted*) *of Ordinary Poultry* (*13*)—Moreover, the authors carried out detective isoaltion of the microbe from fresh excrement of ordinary healthy poultry, but all these examinations were also negative.

The Variation of Vitamin B_{12} *during Incubation of Egg*—Further

TABLE X

Variation of Vitamin B_{12} *during Incubation of Eggs*

Days in incubator Sample	10~12	14	17	19~20
	B_{12} *per cent*	B_{12} *per cent*	B_{12} *per cent*	B_{12} *per cent*
Embryo	10~15	20	50	75~95
Egg yolk	75~70	65	40	20~5
Egg white	15~10	10	10	5~0

studies on biosynthesis of vitamin B_{12} in poultry was carried out by testing the variation of this vitamin during incubation of eggs. (Mikata, Akatsuka and Harashima) The results were calculated by Euglena assay. (Table X).

As seen in the above results, vitamin B_{12} in egg may be transferred to embryo on nuclear metabolism, but no variation of this vitamin in total amount was found during the development of embryo. From the above experiments, it may be considered that poultry has no synthetic ability of this vitamin.

EXPERIMENT 4. INSECTS

In the 4th experiment, the authors (Sahashi and Takahashi) (*14*) attempted to study vitamin B_{12} metabolism in insects and the source of the vitamin in the body. At first, the vitamin B_{12} content in Japanese silk-worm was estimated by the ordinary Euglena method, and on the other hand, the presence of vitamin B_{12} in mulberry-leaves was accurately tested. After repeating careful analysis such as paper chromatography, bioautography and alkali degradation of the vitamin B_{12} concentrated fraction, the occurrence of vitamin B_{12} in larva, pupa and moth were determined in considerable amount, but mulberry leaves were always negative. The experimental data may be briefly given as follows:

TABLE XI

The content of Vitamin B_{12} in Egg, Larva, Pupa and Moth

Date of sampling	Sample	Each individual weight (fresh)	Water content	V. B_{12} (fresh)	V. B_{12} body per 100 bodies
	g.	*g.*	*per cent*	*mγ/100g.*	*mγ*
Egg (Sept. 15 '52)	10.01	6.00×10^{-4}	66.26	2.0	trace
Larva (Jul. 28 „)	56.01	3.73	80.15	2.0	75
Pupa (Aug. 1 „)	11.99	1.20	78.33	3.0	36
„ („ 4 „)	11.55	1.16	78.05	3.2	37
Moth („ 12 „)	7.30	0.61	73.52	2.7	17

From these findings, vitamin B_{12} in silk-worms may be supposed to be produced in vivo. In respect to the vitamin B_{12}-forming mechanism in the living body, studies will be further continued.

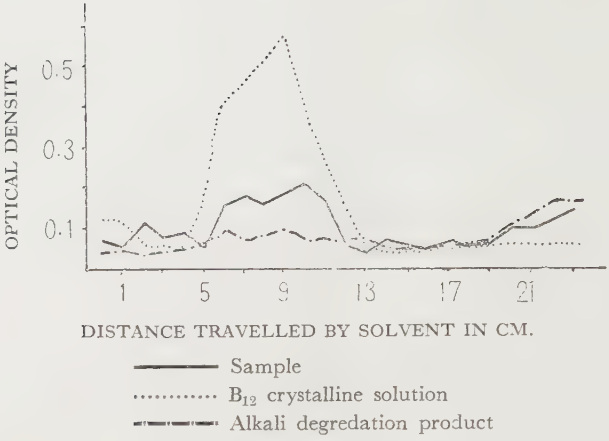


FIG. 4. Bioautogram of egg concentrate

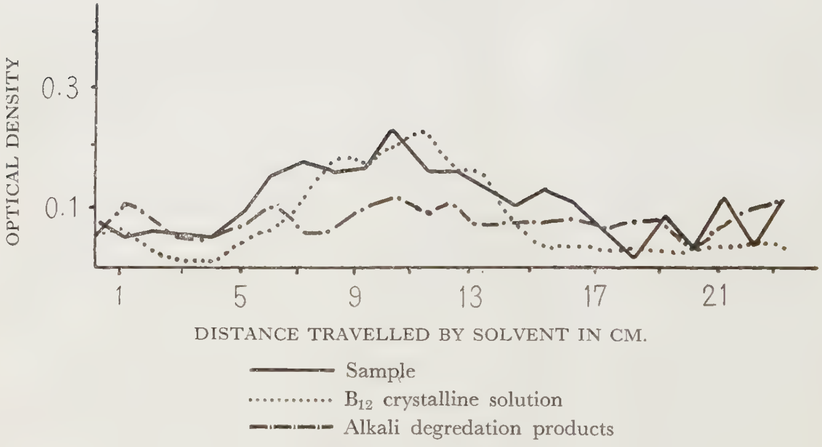


FIG. 5. Bioautogram of pupa concentrate

EXPERIMENT 5. MICROORGANISMS AND VITAMIN B_{12} FORMING PRECURSOR

In 1950, the authors (Sahashi, Mikata and Sakai) reported studies on biochemical synthesis of vitamin B_{12} with incubation of 5,6-

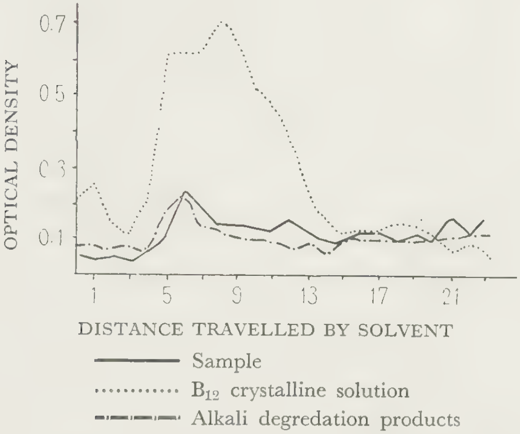


FIG. 6. Bioautogram of mulberry-leaf concentrate purified by benzylalcohol

dimethylbenzimidazole to the broth for *Streptomyces* or fresh liver paste, and stated that the increase of vitamin B_{12} was observed in the experiment.

In 1949, one of the authors (Sakai in Sakaguchi Laboratory, University of Tokyo) isolated a new active strain of *Streptomyces* from the pasture in Chiba and Hokkaido as described above.

In 1950, also Sakai isolated several new strains belonging to *Actinomycetes* (C. R. 21, Sakai; P. D. 61, Sakai) from cow-rumen and poultry-droppings, but these microbes differed from the ordinary *Streptomycin*-producing strains.

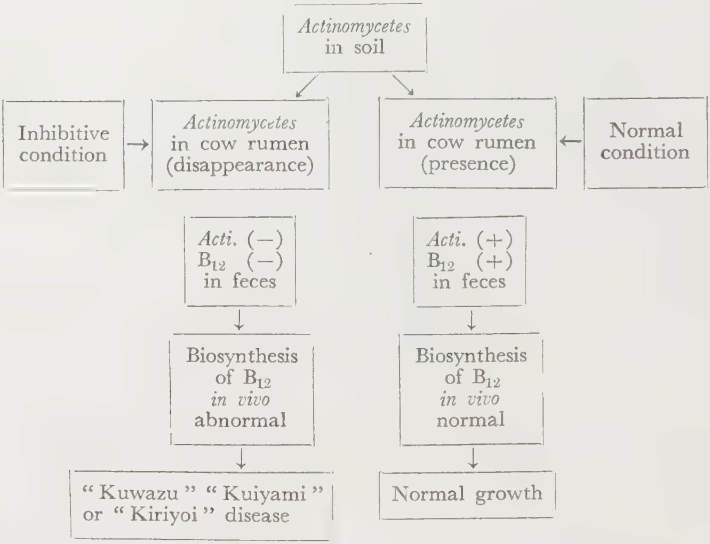
Afterwards, Sakai repeated cultural experiments of the new strains (C. R. 21; P. D. 61) with incubation of 5,6-dimethylbenzimidazole, and succeeded in obtaining 13500 units/ml. vitamin B_{12}-containing broth. On the other hand, after subsequent purification, one of the authors (Mikata and Miki) (*16*) prepared cyrstalline vitamin B_{12a}, indicating absorption maximum at 351 mμ.

From the above results, the new strains isolated from cow-rumen and poultry-droppings may belong to vitamin B_{12}-forming *Actinomycetes*.

SUMMARY AND DISCUSSION

(1) The biosynthesis in vivo of vitamin B_{12} seems to take place according to the following schema, and vitamin B_{12}-deficiency such as

" Kuwazu," " Kuiyami " and " Kiriyoi " diseases among cattle in Japan may be due to the absence of vitamin B_{12} producing *Actinomycetes* in vivo caused by certain inhibitive condition.


Actinomycetes in soil
Inhibitive condition
Actinomycetes in cow rumen (disappearance)
Actinomycetes in cow rumen (presence)
Normal condition
Acti. (−) B_{12} (−) in feces
Acti. (+) B_{12} (+) in feces
Biosynthesis of B_{12} *in vivo* abnormal
Biosynthesis of B_{12} *in vivo* normal
" Kuwazu " " Kuiyami " or " Kiriyoi " disease
Normal growth

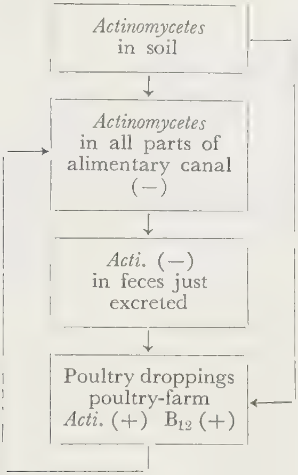
Considerable kinds of elements or compounds such as SiF_6^{--} ions in nature may be physiological inhibitors for vitamin B_{12}-production in various organisms as seen in the case of " Kuwazu disease."

(2) Complete nutrition of Vitamin B_{12} in rats was observed even when fed on the vitamin B_{12}-deficient diet. No effect of vitamin B_{12} on the analytical value of vitamin D was observed by means of bioassay with rats. No anemia was also recognized in rats in any case of the experiments with completely vitamin B_{12}-free diets. Hence, the authors considered that vitamin B_{12} in rats may be produced in vivo.

(3) The relation between the vitamin B_{12} in poultry droppings and *Actinomycetes* may be considered as follows:

In poultry droppings, vitamin B_{12}-producing microorganisms belonging to *Actinomycetes* were always positively confirmed, but in any part of alimentary canals of poultry, no *Actinomycetes* was ever detectable. Hence, vitamin B_{12} in poultry droppings seems to be produced after excretion.

(4) Vitamin B_{12} in silk-worms may be considered to be produced in vivo. Vitamin B_{12} was always detectable in egg, larva, pupa and


Actinomycetes in soil
Actinomycetes in all parts of alimentary canal (−)
Acti. (−) in feces just excreted
Poultry droppings poultry-farm *Acti.* (+) B_{12} (+)

moth, but on the contrary, completely negative in mulberry-leaves. Further investigation of vitamin B_{12}-forming mechanisms in other insects will be published later.

The authors wish to express their sincere thanks to Prof. Y. Kuno (Chairman, Committee on Vitamin B research) and Prof. M. Shioiri (President, Shiga Agricultural College) for their kind guidance. They are also indebted to Drs. K. Masui, K. Sakaguchi, K. Inoue, S. Uchino and H. Matsumuro for their cordial advice. The cost of this research has been defrayed from the Department of Education to which the authors' thanks are due.

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The Journal of Biochemistry, Vol. 40, No. 3.

EFFECT OF VITAMIN B_{12} ON METABOLISM. I

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(Received for publication, February 20, 1953)

INTRODUCTION

Recent advance in biochemistry has informed that vitamin B_{12} may be essential to the formation of nucleic acid and nucleoprotein *in vivo*. In 1939, Lipmann (*1*) already reported that formative relation *in vivo* of vitamin B_1 and pyruvate metabolism in animal body may be as in the following schema:

$$\begin{tikzcd}
\text{Pyruvate} \arrow[r] \arrow[d] & B_1\text{-enzymatic system} \\
CH_3COOH+CO_2 & B_1+ATP \arrow[u]
\end{tikzcd}$$

In 1950, R. W. Vilter (*2*) reported studies on the relations of vitamin B_{12}, folic acid, thymine, uracil and methyl group donors in persons with pernicious anemia and related diseases.

In the same year in Japan, further confirmation for these researches was made by S. Ito (*3*), University of Nagoya and a distinct increase of ATP was observed in the case of bone marrow culture.

Afterwards, in the same year, N. Sakurai (*4*) reported several excellent hematological researches of energy metabolism in race-horses, and macrocytic anemia was observed in animals after energy work, while there was an increase of red corpuscles at rest. However, in his publication, no further studies were made from the view-point of vitamin-biochemistry. Then the present authors (*5, 6*) have recently attempted to study the effects of vitamin B_{12} on energy metabolism as follows:

Experiment 1. Variation of Vitamin B_1 in Blood of Cattle Put on a Tread-mill—In carrying out this experiment, a holizontal tread-mill was first adjusted to 0.75 meters per second in velocity. Two young cattles (♀ 2 years of age, non-identical twins) were used: One cattle (A) was put on the tread-mill, carrying a load (20 kg.) and restless walking was continued for 4.5 hours, and the variation of vitamin B_1 content in blood was estimated during this energy work. The other one (B) was kept in a shed at rest and served as negative control. After repeating the experiment, 28 (mean) per cent decrease of vitamin B_1 in blood was observed in animal (A group) at the end of experiments and 13 per cent control.

Experiment 2. Variation of the Red Blood Cells in Cattle Put on a Treadmill Adjusted to 6° in Acclivity and 0.75 Meters per Second in Velocity—The above tread-mill used in the 2nd experiment was again adjusted to 6° in acclivity and animals (A, B) were alternately used for the following experiments. One was placed on the same condition carrying a load (20 kg.) and restless working 0.75 meters/sec. in velocity for 4.5 hours, and the other serving as negative control.

TABLE I

Variation of Vitamin B_{12} Content in Energy Metabolism

		Before Experiment	After Experiment
Expt. 1.	Expt. animal A	$770\times 10^4/\overline{mm.}^3$	$660\times 10^4/\overline{mm.}^3$
	Control B	790	780
Expt. 2.	Expt. animal B	692	530
	Control A	675	961
Expt. 3.	Expt. animal A	956	649
	Control B	811	820
Expt. 4.	Expt. animal B	635	580
	Control A	777	830

From the above result, it was found that the animals put on the tread-mill indicated severe symptom of macrocytic anemia.

Experiment 3. Variation of Vitamin B_1 and B_{12} in Blood of Cattle Put on the Above Treadmill—The variation of vitamin B_1 and B_{12} content in blood was estimated. 31.2 per cent decrease of vitamin B_1 in blood was observed in experimental animals at the end of experiments and 4.7 per cent in control. Estimation of vitamin B_{12}: To 5 ml. blood from jugular vein was added 10 mg Na-citrate and the the solution was treated with 1 per cent solution of papain-diastase (2:1) at 37 for 44 hours. After removing protein with 10 per cent metaphosphoric acid, vitamin B_{12} in blood was determined by Euglena bioassay. Vitamin B_{12} in blood of animals put on energy work indicated a decrease, but results in animals at rest showed rather an increase.

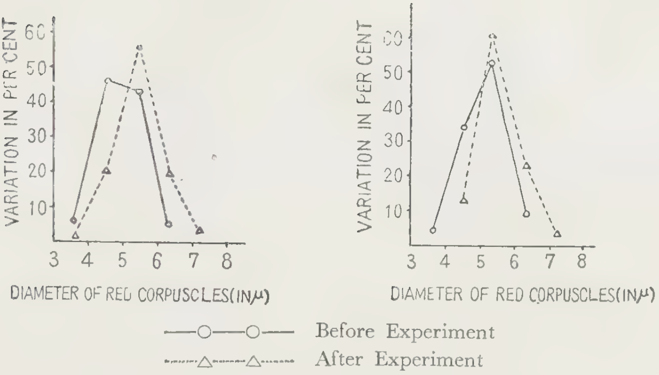


FIG. 1. Variation of the red blood cell counts and of the diameter of red corpuscles.

TABLE II

Variation of Vitamin B_{12} Content in Energy Metabolism.

		Before Expt.	After Expt.	Per cent of variation
		mγ per cent	*mγ per cent*	
Expt. 1.	Expt. animal A	65	53	−18.5
	Control B	67	68	+1.5
Expt. 2.	Expt. animal B	82	67	−18.3
	Control A	75	80	+6.7

Experiment 4. *Variation of Vitamin B_1 and B_{12} in Blood Effected by Energy Metabolism after Injections of Vitamin B_1*—Cattle put on the above tread-mill adjusted to 6° in acclivity was previously treated with 50 mg. vitamin B_1 by subcutaneous injection at the beginning of experiment, and carrying a load (20 kg.) and restless walking was continued for 4.5 hours so mentioned above. At the end of experiment, variation of vitamin B_1 and B_{12} in blood was determined under the same condition as described above. The results gave 18.5 per cent decrease of vitamin B_1 in blood which corresponded to 10 per cent less decrease compared with 28.6 per cent decrease in the case of untreated cattle (Table III).

Moreover, in the present experiment, vitamin B_{12} in blood showed 3.9 per cent decrease which is also much lower than the decrease in blood of the untreated animals (Table IV).

Experiment 5. *Variation of Vitamin B_1 and B_{12} in Blood of Cattle after Injections of Vitamin B_1 and B_{12}*—Cattle were kept under the same condition carrying a load (20 kg.) and restless walking as mentioned above. At first, animals were treated with 30 γ crystalline vitamin B_{12} solution by subcutaneous injection, and after 2 days, injections of 50 mg. vitamin B_1 were administered at the beginning of experiment. At the end of experiment, variation of vitamin B_1 in blood indicated only 8.7 per cent decrease, which corresponded to the content of vitamin B_1 in cattle kept at rest.

TABLE III

Variation of Vitamin B_1 in Blood Effected by Energy Metabolism after Injection of Vitamin B_1 and B_{12}

	Total amount of vitamin B_1 after energy metabolism
	per cent
I. Untreated animal	−28.6
II. Animal treated by vitamin B_1 only	−18.5
III. Animal treated by vitamins B_1 and B_{12}	−8.7

TABLE IV

Variation of Vitamin B_{12} in Blood effected by Energy Metabolism after Injection of Vitamin B_1 and B_{12}

	Total amount of vitamin B_{12} after energy metabolism
	per cent
I. Untreated animal	−18.4
II. Animal treated by vitamin B_1 only	−3.9
III. Animal treated by vitamins B_1 and B_{12}	−2.1

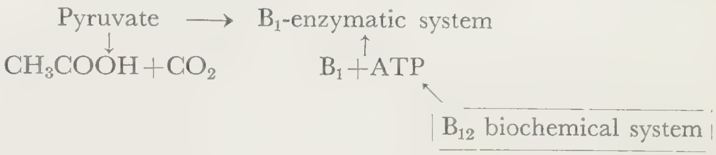
SUMMARY AND DISCUSSION

1. The total amount of vitamin B_1 and B_{12} decreased in blood on energy metabolism. The red blood cell counts in animals put on energy work also indicated a decrease and the diameter of red corpuscles increased, as seen in macrocytic anemia—typical symptom of vitamin B_{12}-deficiency.

2. The decrease of vitamin B_1 and B_{12} on energy work was completely protected by prophylactic injection of the vitamins at the begin-

ning of experiment. Therefore, the relation between vitamins B_1 and B_{12} on energy metabolism may be always extremely intimate.

From these findings, vitamin B_{12} seems to be essentical to energy metabolism in the following relation:



The authors wish to express their sincere thanks to Prof. Y. Kuno (Chairman, Comittee on Vitamin B Research) and Prof. M. Shioiri (President of Shiga Agricultural College) for their kind guidance. They are also indebted to Drs. K. Inoue and S. Uchino for their cordial advice. The cost of this research has been defrayed by grants from the Department of Education to which the authors' thanks are due.

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The Journal of Biochemistry, Vol. 40, No. 3.

STUDIES ON THE CONJUGATED LIPIDS. V.

ON THE ENZYMATIC HYDROLYSIS OF CEREBRON

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(Received for publication, February 23, 1953)

In previous communication the author has reported that ceramide, phosphoric acid and choline were obtained by the enzymatic hydrolysis of sphingomyelin (*1*). This result of the enzymatic decomposition together with the physiological or chemical structural relation between sphingomyelin and cerebroside seemed to suggest that these two substances might be interchangeable from one to another in the animal body through ceramide as follows:

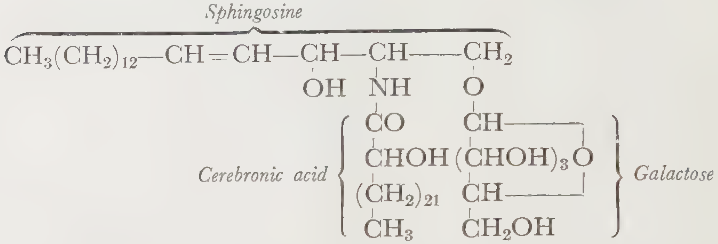
$$\begin{tikzcd}
 & \begin{array}{c}\text{Sphingosine-Fatty acid}\\ \text{(Ceramide)}\end{array} \arrow[dl, dashed, shift left] \arrow[dr, dashed, shift left] & \\
\begin{array}{c}\text{Sphingosine-Fatty acid-Phosphorylcholine}\\ \text{(Sphingomyelin)}\end{array} \arrow[ur, shift left] & & \begin{array}{c}\text{Sphingosine-Fatty acid-Sugar}\\ \text{(Cerebroside)}\end{array} \arrow[ul, shift left]
\end{tikzcd}$$

Based on this assumption, the author has attempted this time to work on the enzymatic action on cerebron, a well-known typical cerebroside, to elucidate the process of its decomposition.

The enzymatic hydrolysis of cerebroside was studied by Rosenheim (*2*), Jungmann and Kimmelstiel (*3*), although they did not get satisfactory results. In 1936, Thannhauser and Reichel (*4*) observed for the first time that galactose was liberated from the cerebroside molecule by the enzyme action, whereby they made use of SH-compounds as activators. They showed at the same time that these activators inhibited the action of sphingomyelinase.

Considering the chemical structural formula of cerebron as indicated below, it seems to us that at least the following three processes in the enzymatic decomposition of cerebron are possible: (a) the galactosidic linkage of galactose to sphingosine may be split; (b) the acid-amidic linkage of cerebronic acid to sphingosine may be attacked; (c) these two processes may occur simultaneously.

Structure of Cerebron (*5*)



The first type of decomposition mentioned above was observed by Thannhauser and Reichel (*4*), however, they did not further refer to the question whether or not the acid-amidic linkage between fatty acid and sphingosine is broken down. In order to clarify this point, the author first isolated cerebron and subjected to the enzymatic decomposition under the experimental conditions of Thannhauser and Reichel (*4*). He has determined glactose and amino-N which would be freed, and studied other substance which would be obtained in this enzymatic decomposition.

EXPERIMENTAL

Isolation of Cerebron from Protagon—100 g. of protagon, which had been obtained from pig brain in the usual manner, were dissolved in 500 ml. of purified pyridine at 60°, cooled overnight at 0°, and the precipitate formed was filtered off. By evaporating the mother liquor under vacuum, a residue, the cerebroside fraction, was obtained, which was recrystallized from the mixed solution of chloroform and methanol (3:1). This procedure of recrystallization was repeated over and over again whereby cerebroside powder, which was at first a little yellowish, became gradually white and rustling. Finally snow white crystal of cerebroside were obtained, yield of which was about 1.7 g. The result of analysis showed that this substance accorded with cerebron. Since a minute amount of kerasin, if it existed, could have no essential influence on the present experiment, this substance was regarded as cerebron in this communication, and the theoretical values were calculated as cerebronylderivatives.

Analysis of Cerebron: $C_{48}H_{93}NO_9$

N:	Found	1.70%
	Calcd.	1.69%

(Substance: 42.50 mg.; *N*/50 NaOH: 2.60 ml.)

Preparation of Substrate Solution—0.460 g. of cerebron were dissolved in 5 ml. of 95 per cent ethylalcohol by heating. This solution was poured into ten volumes of the distilled water warmed to 80°, and heated on the water bath to evaporate ethylalcohol. The evaporation was continued on the water bath with occasional adding of a small amount of distilled water, until ethylalcohol was thoroughly removed. Total volume was then made up to 50 ml. with distilled water and the solution of cerebron thus obtained was stored in a cool and dark chamber under a layer of toluene. This colloidal solution was stable for a long storage.

Preparation of Enzyme Solution—Enzyme solution was prepared after the method used in obtaining the solution of sphingomyelinase (*1*). Fresh spleen of calf was cut in slices, frozen in a mixture of ether and solid carbondioxide, dried completely in a vacuum dessicator, and then pulverized. 3 g. of this fine powder were suspended in 100 ml. of physiological saline solution and were shaken vigorously for eight hours. The colloidal solution was transfered to a collodium bag and dialyzed against distilled water for a few days, until the ferricyanide-reducible substances had been removed entirely. The liquid in the bag was centrifuged, and then stored in the ice chamber as the enzyme solution, a small amount of toluene being added to. Prior to starting experiment, this enzyme solution was activated by passing through hydrogen sulfide gas, which had been found to be the most effective activator for cerebrosidase (*4*).

Determination of Enzymatic Activity—Enzymatic hydrolysis was carried out in the reaction mixture composed of following solutions in the proportion indicated: substrate solution, 5 parts; enzyme solution, 1 part; buffer solution, 3 parts; toluene, a small amount (for the prevention of an undesireable decomposition). This reaction-mixture was stoppered and held at 38° in an incubator. Complete hydrolysis was attained in twenty four hours. After incubation, an aliquot of this mixture was taken out and warmed on the water-bath so that hydrogen sulfide was completely removed. Galactose which was liberated during above hydrolysis was estimated by the method of Hanes (*6*), and free amino-N by the method of van Slyke. The enzyme solution, when incubated as described above, produced ferricyanide-reducible substances to some extent. It was therefore necessary to subtract the autolysis values of enzyme solution from those of enzyme-substrate mixture.

Optimal Conditions of Enzymatic Activity—The effects of hydrogen ion concentration and of hydrogen sulfide were first investigated. As buffers were used bicarbonate-citric acid buffer of McIlvane for the acidic range, and hydrogen chloride-veronal buffer of Michaelis for the alkaline range, respectively. Hydrogen sulfide was introduced for one hour into the enzyme solution. The enzymatic activity was examined in the way to observe the increase of galactose content in 9 ml. of the mixed solutions of enzyme, substrate and buffer. If hydrolysis were complete, 9 ml. of the reaction-mixture would liberate theoretically 1 mg. of galactose, corresponding to 1.23 ml. of *N*/50 sodium thiosulfate. The experimental results are shown in Table I.

It is obvious that the enzyme action is greatly influenced by the use of activator, H_2S. However, when hydrogen sulfide was used, the hydrolysis was complete at pH 7.0. Thannhauser and Reichel (*4*) reported that cerebroside was hydrolyzed almost completely at pH 4.9, although the present author found that the enzymatic activity was only slight at pH 5.0.

TABLE I

Effect of Hydrogen Ion Concentration and Hydrogen Sulfide on the Enzymatic Activity

(38°, 24 hours)

pH		Without H_2S		With H_2S	
		$N/50$ $Na_2S_2O_3$	Grade of hydrolysis	$N/50$ $Na_2S_2O_3$	Grade of hydrolysis
		ml.	*per cent*	*ml.*	*per cent*
McIlvane buffer	5.0	0.08	6.5	0.17	13.8
	7.0	0.12	9.8	1.25	101.6
Michaelis buffer	8.0	0.08	6.5	0.25	20.3
	9.0	0.08	6.5	0.08	6.5
Theoretical value in case of complete hydrolysis		1.23	100.0	1.23	100.0

Hydrolysis of Cerebron—In accordance with the results above described, cerebron was hydrolyzed under the following conditions, and then galactose and amino-N liberated in the reaction-mixture were determined, the results are shown in Table II.

TABLE II

Enzymatic Hydrolysis of Cerebron

(pH 7.0; 38°; Activator, H_2S; 24 hours)

	Observed	Calculated	Liquid amount tested
	ml.	*ml.*	*ml.*
$N/50$ $Na_2S_2O_3$ (galactose)	2.48	2.46	18
N_2 (amino-N)	0	0.53	18

Although galactose was completely liberated, the liberation of amino-N was not observed, showing that the galactosidic linkage of galactose to sphingosine was broken down, whereas the acid-amidic linkage of fatty acid to sphingosine would not. The product formed in this enzymatic hydrolysis must be, accordingly, galactose and cerebronylsphingosine. Though the former could not be identified owing to its insufficient amount to form glalactosazone, the existence of the latter was confirmed as described below.

Isolation of Cerebronylsphingosine Fraction—150 ml. of the reaction-mixture which had been hydrolyzed enzymatically were condensed to about 20 ml. under reduced pressure, added to a large amount of acetone, and cooled overnight at 0°. The precipitate formed was filtered, dissolved in warm acetone, and filtered through a warmed filter. After the filtrate was evaporated to dryness, white powder was obtained which was purified by repeating the above procedures. About 50mg. of cerebronylsphingosine was finally obtained as snow white powder.

Analysis of Cerebronylsphingosine: $C_{42}H_{83}NO_4$

N: Found 2.18%
Calcd 2.13%
(Substance: 35.70 mg.; $N/50$ NaOH: 2.80 ml.)

The chemical analysis of the other elements could not be carried on as the material was not sufficient enough.

SUMMARY

Cerebron being decomposed enzymatically under adequate conditions, galactose was liberated completely from the cerebron molecule and the formation of cerebronylsphingosine was recognized, whereas fatty acid was not liberated. An assumption may be presented therefore that the enzymatic hydrolysis of cerebron proceeds along the following scheme:

$$\text{Cerebron} \longrightarrow \begin{cases} \text{Galactose} \\ \text{Cerebronylsphingosine} \end{cases}$$

The author wishes to express his deep indebtness to Prof. Morio Yasuda for advice and encouragement for this work. This work was supported by the grant of the National Research Council of Japan.

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The Journal of Biochemistry, Vol. 40, No. 3.

METABOLISM OF HISTIDINE. II.

MERCURY, CADMIUM OR ZINC, CO-FACTOR OF L-HISTIDINE DEAMINASE

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(Received for publication, March 9, 1953)

In the former report (*1*) it was mentioned that urocanic acid is an intermediate on the metabolic pathway of L-histidine. Recently, utilizing labelled L-histidine, Hayaishi *et al.* (*2*) and Borsook *et al.* (*3*) observed successively the conversion of L-histidine to urocanic acid, the former with cell-free extract of L-histidine-adapted bacteria, the latter with homogenate of rabbit liver. Now it becomes beyond doubt that the decomposition of L-histidine is primed with the liberation of its α-amino group.

Concerning the mechanism of this one-step enzyme reaction, Ichihara *et al.* (*4*) reported that folic acid, glutathione and ascorbic acid act as the co-factors of L-histidine deaminase obtained from rabbit liver.

In this paper, the purification of L-histidine deaminase from bacterial origin and the participation of metal ions, Hg^{++}, Cd^{++} and Zn^{++}, in the deamination catalyzed by this enzyme are submitted.

MATERIALS AND METHODS

Analytical Procedures—L-Histidine was determined by Sera's bromine reaction (*5*), urocanic acid by Sera's diazo reaction (*5*), liberated ammonia by Conway's microdiffusion method and protein by Folin-Ciocalteau's phenol reagent (*6*).

Preparation of Acetone Powder of L-Histidine-adapted Cells—A soil bacteria which can actively utilize L-histidine was grown on the plate agar consisting of 1 per cent bouillon, 1 per cent polypeptone, 1 per cent dried brewers' yeast, 0.2 per cent NaCl and 3 per cent agar, at 37° for 15 hours. Then, bacteria cells were harvested, suspended in twenty volumes of $M/15$ phosphate buffer (pH 7.4) containing 2 mg. per ml. of L-histidine, and fully adapted to L-histidine by shaking at 37° for about 100 minutes. Thereafter, L-histidine-adapted cells were centrifuged and dried with ice-cold acetone.

RESULTS

Purification of L-*Histidine Deaminase*—

(1) Extraction of the enzyme: Suspended in fifty volumes of *M*/15 phosphate buffer (pH 8.0), acetone powder of histidine-adapted cells was left overnight in refrigerator, then centrifuged at 5,000 r.p.m. for 30 minutes, and the cell debris was discarded.

According to one of the experimental data, shown in Table I, the volume of this extract from 2 g. of acetone powder was 90 ml. and contained 279 mg. of protein, its specific activity being 860. Yield of the enzyme may be expressed as 100 per cent in this case.

(2) Removal of nucleic acid: Protamine sulfate, about 2 mg. per ml. of the extract, was dissolved in a small amount of water, and was added into the cell-free extract, with continuous stirring. Fibrous precipitate produced was centrifuged, washed with a small quantity of buffer, then discarded.

Volume of the combined supernatants was 101 ml., total protein diminished down to 202 mg., specific activity rose up to 1,140. Yield was 96 per cent.

(3) Heat treatment: This nucleic acid-free enzyme solution was added with 10 per cent (*w*/*v*) of caolin and heated at 60° for 15 minutes, and centrifuged. The precipitate was discarded after washing with a small amount of buffer.

The combined supernatants were 97 ml. in volume containing 107 mg. of protein. The specific activity was raised to 1,900. Yield was 85 per cent.

(4) Adsorption and elution on / from tricalcium phosphate gel: The heat-treated enzyme solution was adsorbed at pH 5.7 with freshly prepared 10 per cent (wet *w*/*v*) tricalcium phosphate gel. The gel which adsorbed enzyme protein was centrifuged, washed once with distilled water, then eluted with *M*/15 borate buffer (pH 8.3). Elution was performed twice, each for 30 minutes.

Total protein contained in this fraction fell down to 7.5 mg., and the specific activity increased remarkably up to 13,600. Yield was 42.5 per cent. Though highly purified, it was contaminated still by urocanic acid-decomposing enzyme, so another heat treatment was performed.

(5) Heat treatment: Without adding caolin, the enzyme solution was heated at 60° for 15 minutes and centrifuged at 20,000 r.p.m. for 15 minutes. Through this treatment, increment of specific activity

was not detectable but the perfect elimination of urocanic acid-decomposing enzyme was attained.

TABLE I

Purification of the Enzyme

	Volume	Total protein	Specific activity*	Yield
	ml.	*mg.*		*per cent*
(1)	90.0	279.0	860	100.0
(2)	101.0	202.0	1,140	96.0
(3)	97.0	107.0	1,900	85.0
(4)	52.0	7.5	13,600	42.5
(5)	50.0	4.2	13,500	23.5

0.5 ml. of the enzyme solution of each step of purification was incubated with $5 \times 10^{-3} M$ histidine in $10^{-1} M$ borate buffer (pH 8.3) at 37° for 1 hour. Total volume was 4 ml.

* Specific activity: the amounts (γ) of L-histidine decomposed per mg. of protein included in the enzyme solution at 37° for 1 hour.

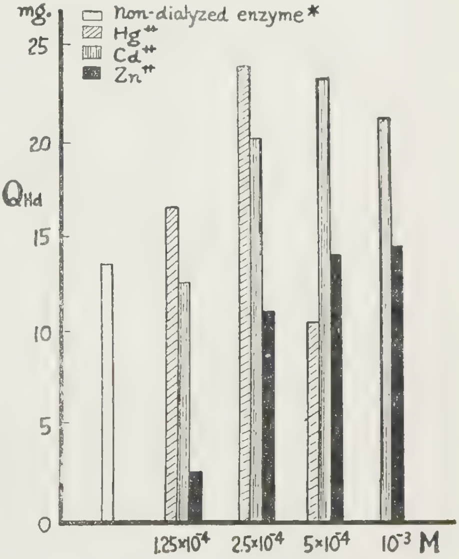

FIG. 1. QHd shows mg. of histidine decomposed per mg. of protein in the enzyme solution at 37° for 1 hour.

Each reaction mixture (4 ml.) contained $10^{-1} M$ borate buffer (pH 8.3), $5 \times 10^{-3} M$ histidine, 1.25×10^{-4}, 2.5×10^{-4}, 5×10^{-4} and $10^{-3} M$ of Hg^{++}, Cd^{++} or Zn^{++} and 0.5 ml. dialyzed enzyme solution containing 40 γ of protein.

* Activity of non-dialyzed enzyme was 13,500 as shown in Table I and it disappeared perfectly through dialysis.

Using this solution of L-histidine deaminase, following experiments were carried out.

Effects of Inhibitors—Among various metal ions, Mn^{++}, Fe^{+++}, Ba^{++}, Ag^{+} and Pb^{++} exhibited perfect inhibition in concentration of 2×10^{-4} *M*. 10^{-2} *M* and 2×10^{-2} *M* cyanide inhibited 54 per cent and 97 per cent of the deamination reaction, respectively. The inhibitory action of 8-oxyquinoline was 40 per cent in concentration of 10^{-3} *M*. 10^{-3} *M* of o-phenanthrolin, $5\times10^{\ 3}$ *M* of semicarbazide and 2×10^{-2} *M* of arsenate had no effects on the enzyme reaction.

Inactivation through Dialysis and Reactivation by Hg^{++}, Cd^{++} *or* Zn^{++}—The purified L-histidine deaminase was inactivated through dialysis against a large volume of deionized water overnight. This inactivated enzyme regained its activity when Hg^{++}, Cd^{++} or Zn^{++} of appropriate concentrations, as shown in Fig. I, was added.

The optimal concentration of Hg^{++} was 2.5×10^{-4} *M* and that of Cd^{++} was 5×10^{-4} *M*. The extent of activation by Zn^{++} was not so remarkable as Hg^{++} and Cd^{++}.

SUMMARY

1. L-Histidine deaminase was highly purified and completely freed of urocanic acid-decomposing enzyme.

2. Hg^{++}, Cd^{++} or Zn^{++} participates the enzymatic reaction as a co-factor.

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STUDIES ON CARBOXYPEPTIDASE II.

THE ACTION OF CARBOXYPEPTIDASE ON α-BROMOISOBUTYRYL-*o*-, -*m*- AND -*p*-AMINOBENZOIC ACIDS

By TAKEO SAITO AND KEIICHI SAITO

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(Received for publication, March 17, 1953)

In our laboratory Utsunomiya (*1*) investigated the action of carboxypeptidase on chloroacetyl, α-bromo-propionyl and α-bromo-isocapronyl derivatives of *o*- ,*m*- and *p*-aminobenzoic acids and found that *m*- and *p*-aminobenzoic acids derivatives are capable of being split by carboxypeptidase, while *o*-compounds are quite resistant to it. The present paper is a supplement to this investigation and reports on the action of carboxypeptidase on α-bromoisobutyryl derivatives of *o*-, *m*- and *p*-aminobenzoic acids.

MATERIALS AND METHODS

Substrates—α-Bromoisobutyryl-*o*-, -*m*- and -*p*-aminobenzoic acids employed as substrates were prepared from α-bromo-isobutyryl-bromide and aminobenzoic acids as described by one of the authors (Keiichi Saito, (*2*)).

The ortho compound melted at 148° and gave, N (micro-Kjeldahl method), 4.79 per cent (calculated for $C_{11}H_{12}NO_3Br$, N, 4.89 per cent). The meta compound melted at 173° and gave, N, 4.55 per cent (calculated for $C_{11}H_{12}NO_3Br$, N, 4.89 per cent). The para compound melted at 223° and gave, N, 4.64 per cent (calculated for $C_{11}H_{12}NO_3Br$, N, 4.89 per cent).

Enzymes—Carboxypeptidase and enterokinase were prepared in the same manner as described by Utsunomiya (*1*). It's action on chloracetyl tyrosine, chloroacetyl-*m*- and -*p*-aminobenzoic acids were tested and it was found that the activity of the enzyme was approximately the same as that employed by Utsunomiya.

The experimental procedure was exactly the same as that reported by Utsunomiya. Double control experiments were performed with a special precaution, by the use of enzyme and buffer on the one hand, and by the use of substrate and buffer on the other. These values were subtracted from that of the main experiment.

EXPERIMENTAL RESULTS

Fig. 3 represents the hydrolysis rates of *m*- and *p*-compounds at pH

8.4. Just as in the case of other halogen acyl aminobenzoic acids derivatives, so also in this case only *p*- and *m*-compounds undergo hydrolysis, while *o*-compound is scarecely split by the enzyme. The results of the experiments are recorded in Figs. 1 and 2.

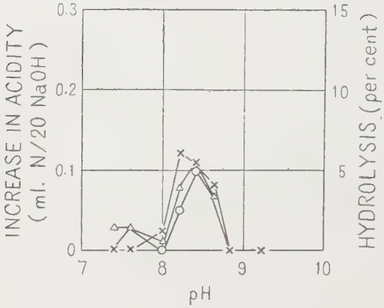

Fig. 1. Action of carboxypeptidase on α-bromoisobutyryl-*m*-aminobenzoic acid at varying pH.

(Increase in acidity after 8, 24 and 48 hours. Temperature=30°.)

——×——×—— 48 hrs. ——△——△—— 24 hrs. ——○——○—— 8 hrs.

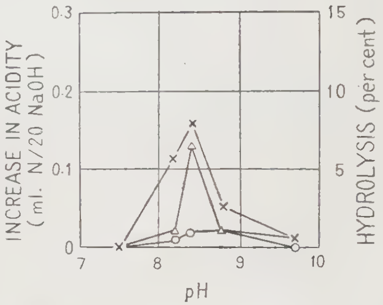

Fig. 2. Action of carboxypeptidase on α-bromoisobutyryl-*p*-aminobenzoic acid at varying pH.

(Increase in acidity after 8, 24 and 48 hours. Temperature, 30°.)

——×——×—— 48 hrs. ——△——△—— 24 hrs. ——○——○—— 8 hrs.

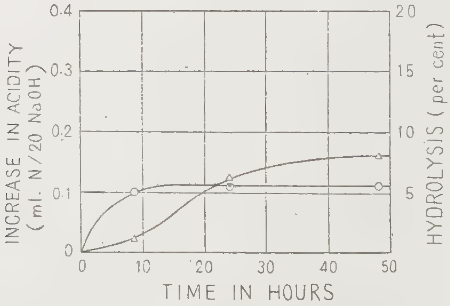

Fig. 3. Hydrolysis rates of α-bromoisobutyryl-*m*- and -*p*-aminobenzoic acids by carboxypeptidase. (Temperature, 30°.)

——○——○—— α-Bromoisobutyryl-*m*-aminobenzoic acid (pH=84)

——△——△—— α-Bromoisobutyryl-*p*-aminobenzoic acid (pH=8.4)

From these figures we see that the initial hydrolysis rate of *m*-compound is greater than that of *p*-compound, the optimum pH being found in both cases at pH 8.4. Moreover a comparison of these results with those of other halogen acyl derivatives reported by Utsunomiya leads to the conclusion that the hydrolysis of α-bromoisobutyryl-aminobenzoic acids by means of carboxypeptidase proceeds at a slower rate than other halogen acyl compounds and with nearly the same speed as in the case of α-bromoisocapronyl compounds.

SUMMARY

The action of carboxypeptidase on α-bromoisobutyryl-*o*-, -*m*- and -*p*-aminobenzoic acids was studied and a comparison was made of the cleavability of these substances with that of other halogen acyl compounds reported by Utsunomiya.

1. Only *m*- and *p*-compounds were split by carboxypeptidase as in the case of other halogen acyl derivatives.
2. The optimum pH lies in both cases at pH 8.4.
3. α-Bromo-isobutyryl derivatives are attacked by the enzyme with slowness as compared with other halogen acyl compounds and at nearly the same rate as α-bromo-isocapronyl derivatives.

In conclusion the authors wish to express their sincere thanks to Prof. Dr. T. Uchino for his kind suggestion throughout this work.

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STUDIES ON CARBOXYPEPTIDASE III.

THE EFFECT OF THE ADDITION OF VARIOUS SALTS ON CARBOXYPEPTIDASE

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(Received for publication, March 17, 1953)

Sugai (*1*) studied the effect of the addition of various salts on the tryptic activity and pointed out the fact that manganese, iron, cobalt and nickel salts accelerate the action of trypsin, while copper, silver, zinc, mercury and lead salts cause the inhibition of it. Likewise, potassium cyanide and potassium ferrocyanide retard the action of trypsin, whereas other complex iron salts, such as potassium ferricyanide, ferrous ammonium sulfate, ammonium ferric alum and sodium nitroprusside bring about the activation of the enzyme.

The present paper deals with the influence exerted by various salts on the activity of carboxypeptidase. Just as in the case of Sugai's research on the tryptic activity, so also in this case a comparison was made of the effects of salts on the initial hydrolysis rate of chloracetyl tyrosine by means of carboxypeptidase. Moreover, the experiment was performed in the presence of varying amounts of salts as well as at varying pH of reaction mixtures, since the addition of salt may cause the shift of optimum pH.

EXPERIMENTAL PROCEDURE

Substrate—Chloracetyl tyrosine was the product prepared by Utsunomiya (*3*) according to the method of E. Fischer (*2*). It melted at 154° and gave, N (micro-Kjeldahl method), 5.34 per cent (calculated for $C_{11}H_{12}O_4$ NCl, N, 5.44 per cent).

Enzyme—Carboxypeptidase and enterokinase were prepared according to the method described by Waldschmidt-Leitz. The activation of carboxypeptidase was carried out with the addition of an equal bulk of enterokinase solution and by keeping it at 30° for 30 minutes.

A sample of 0.1288 g. (=0.5 millimoles) chloracetyl tyrosine was weighed in a 25.0 ml.-measuring flask and dissolved in 2.10–3.05 ml. of 1 *N* NaOH solution with the addition of 5–10 ml. of distilled water and 2.5 ml. of 1 *M* KH_2PO_4 solution. The required pH of the reaction mixture is attained by changing the amount of 1 *N* NaOH solution from 2.10 to 3.05 ml. The flask was then dipped in a thermostat at 30°.

When the contents of the flask attained the temperature of the bath, 2.5 ml. of salt solution, which had been previously warmed to 30°, and 10 ml. of activated carboxypeptidase solution were added, after which the volume was made up to 25.0 ml. with distilled water. The solution was transferred into a conical flask, a 5.0 ml. of sample was immediately pipetted out from it and titrated. About 3 ml. were employed for pH determination and the rest of the solution was kept in a thermostat at 30°. The titration was performed with $N/20$ NaOH solution by formol titration method. After the lapse of 15, 30 and 60 minutes further samples were taken out from the flask and titrated. Under the present experimental conditions an increase in the acidity of 2.0 ml. of $N/20$ NaOH corresponds to 100 per cent hydrolysis.

Two control experiments, which were carried out using only buffer and enzyme on the one hand and using only buffer and substrate on the other, gave always negligible values.

Upon addition of some kind of salt it was observed that a considerable pH shift in the solution took place. In such cases the buffer was so adjusted by the addition of an appropriate amount of 1 N NaOH solution that the required pH could be attained. In all cases, whenever a turbidity due to the addition of salts was produced, the experiment was not made.

EXPERIMENTAL RESULTS

Control Experiment without Salts—We examined at first the action of carboxypeptidase on chloracetyl tyrosine in the absence of salts and at varying pH of the medium. The results are given in Fig. 1.

Fig. 1 shows us, that the highest hydrolysis rate of chloracetyl tyrosine is attained at pH 7.3–7.4. The velocity of hydrolysis thus measured without salts was taken as a standard in all the following series of experiments.

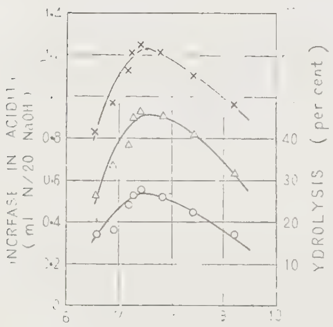

Fig. 1. Action of carboxypeptidase on chloracetyl tyrosine at varying pH.

(Increase in acidity after 15, 30 and 60 minutes. Temperature, 30°)

—○—○— 15 min.
—△—△— 30 min.
—×—×— 60 min.

Effect of NaCl, KCl, NaF, $MgCl_2$, $CaCl_2$, $BaCl_2$ and $AlCl_3$ on the Carboxypeptidase Activity—The influence exerted by these salts was investigated at varying pH of the reaction mixture and at different concentrations of the salts. The relative effects of the salts on the action of carboxypeptidase were conveniently expressed by taking the hydrolysis rate without salts as a standard, as mentioned in the foregoing section, and assigning to it the value 100. The relative effects of the salts thus calculated are contained in the following tables. By reason of the fact that these and all the other salts tested were found to bring about no shift of optimum pH for the action of carboxypeptidase, only the values obtained at about pH 7.4, are recorded in all the following tables.

TABLE I

Influence of NaCl, KCl., NaF, $MgCl_2$, $CaCl_2$, $BaCl_2$ and $AlCl_3$ on the Carboxypeptidase Action

(Control=100)

Salts	Concentration of salts in the reaction mixture	Hpdrolysis rates (*per cent*)			pH	Activation	Inhibition
	M	15 min.	30 min.	60 min.			
NaCl	1×10^{-1}	101.9	105.7	103.3	7.23	—	—
	2×10^{-2}	103.6	100.0	99.2	7.35		
KCl	1×10^{-2}	101.8	103.3	100.0	7.41	—	—
NaF	1×10^{-2}	103.4	101.1	100.9	7.45	—	—
$MgCl_2$	1×10^{-3}	100.0	100.0	100.0	7.36	—	—
$CaCl_2$	2×10^{-3}	105.1	104.3	107.8	7.39	—	—
	1×10^{-3}	100.1	100.0	100.9	7.44		
$BaCl_2$	2×10^{-3}	98.0	94.8	97.3	7.39	—	—
	1×10^{-3}	100.0	100.0	100.9	7.39		
$AlCl_3$	4×10^{-3}	101.8	96.7	100.0	7.49	—	—
	1×10^{-3}	107.1	103.3	100.8	7.48		

Table I shows us clearly that the action of carboxypeptidase is completely unaffected by the presence of these salts.

Effect of Manganese, Iron, Cobalt and Nickel Salts on the Carboxypeptidase Activity.—We then examined the effect of $MnSO_4$, $FeSO_4$, $FeCl_3$, $CoSO_4$, and $NiSO_4$ in the same way as given in the preceding section.

The results are recorded in Table II.

Cobalt and nickel salts exert the most marked accelerating effect on carboxypeptidase. $FeSO_4$ and also $FeCl_3$, though to a less degree,

TABLE II

Effect of $MnSO_4$, $FeSO_4$, $FeCl_3$, $CoSO_4$ and $NiSO_4$ on the Carboxypeptidase Activity

(Standard = 100)

Salts	Concentration of salts in the reaction mixture	Hydrolysis rates (*per cent*)			pH	Activation	Inhibition
		15 min.	30 min.	60 min.			
$MnSO_4$	M 2×10^{-3}	107.1	104.3	100.0	7.37	—	—
	1×10^{-3}	100.0	101.1	101.6	7.35		
$FeSO_4$	4×10^{-3}	136.4	120.4	111.1	7.40	+	—
	1×10^{-3}	129.1	115.1	108.7	7.39		
	2×10^{-4}	109.1	106.5	102.4	7.39		
$FeCl_3$	2×10^{-3}	107.4	102.2	102.4	7.29	+	—
	1×10^{-3}	105.5	105.4	105.6	7.39		
	2×10^{-4}	109.3	104.4	100.9	7.30		
$CoSO_4$	2×10^{-3}	164.3	141.9	133.9	7.55	+++	—
	1×10^{-3}	141.8	133.7	129.4	7.36		
	2×10^{-4}	109.1	108.7	115.1	7.45		
$NiSO_4$	4×10^{-3}	120.0	125.0	120.6	7.32	++	—
	2×10^{-3}	107.1	114.1	111.1	7.36		
	1×10^{-3}	105.4	101.1	110.3	7.35		

show a promoting effect on the enzyme, whereas the action of $MnSO_4$ is quite insignificant.

Effect of Copper, Silver, Zinc, Mercury and Lead Salts on the Carboxypeptidase Activity—In the present section the effects of $CuSO_4$, $AgNO_3$, $ZnSO_4$, $HgCl_2$, and $Pb(C_2H_3O_2)_2$ were studied. The results obtained are given in Table III.

From Table III it follows that copper, zinc and lead salts have no notable action on carboxypeptidase, while silver and mercury salts exert an inhibitory effect on the enzyme. An especially marked inhibition was noticeable in the case of mercury salt.

Effect of KCN and Various Iron Complex Salts on the Carboxypeptidase Activity—Effect of KCN, potassium ferrocyanide, potassium ferricyanide, ferrous ammonium sulfate, ammonium ferric alum, sodium nitroprusside was then examined and the results as shown in Table IV, were obtained.

TABLE III

Effect of $CuSO_4$, $AgNO_3$, $ZnSO_4$, $HgCl_2$ *and* $Pb(C_2H_3O_2)_2$ *on the Carboxypeptidase Activity*

(Standard = 100)

Salts	Concentration of salts in the reaction mixture	Hydrolysis rate (*per cent*)			pH	Activation	Inhibition
		15 min.	30 min.	60 min.			
	M						
$CuSO_4$	4×10^{-3}	100.0	104.3	103.5	7.40	—	—
	2×10^{-3}	109.1	102.2	100.8	7.35		
	1×10^{-3}	101.8	101.1	100.8	7.35		
$AgNO_3$	2×10^{-3}	89.3	83.7	83.3	7.35	—	+
	1×10^{-3}	98.2	98.9	100.0	7.35		
$ZnSO_4$	2×10^{-3}	101.8	101.1	100.0	7.63	—	—
	1×10^{-3}	100.0	101.1	100.0	7.39		
$HgCl_2$	2×10^{-3}	23.2	30.4	35.8	7.35	—	⧻
	1×10^{-3}	90.9	76.9	72.5	7.37		
	2×10^{-4}	98.2	97.8	100.0	7.38		
$Pb(C_2H_3O_2)_2$	2×10^{-3}	98.2	100.0	100.0	7.58	—	—
	1×10^{-3}	98.2	101.1	100.0	7.33		

It is evident from the table that potassium ferricyanide exhibits the most marked accelerating action on carboxypeptidase. Potassium ferrocyanide, ferrous ammonium sulfate and sodium nitroprusside also cause the acceleration of the enzyme to some extent, whereas ammonium ferric alum exerts no effect on carboxypeptidase. Lastly, KCN was found to have a marked inhibitory effect on the enzyme.

Effect of Potassium Permanganate and Hydrogen Peroxide on the Carboxypeptidase Activity—The effect of $KMnO_4$ and H_2O_2 is shown in Table V.

Table V shows that potassium permanganate and hydrogen peroxide have no effect on the action of carboxypeptidase at the concentration tested.

SUMMARY

The effects of various salts on the hydrolysis rate of chloracetyl

TABLE IV

Effect of KCN, $K_4Fe(CN)_6$, $K_3Fe(CN)_6$, $FeSO_4(NH_4)_2SO_4$, $NH_4Fe(SO_4)_2$ *and* $Na_2Fe(CN)_5NO$ *on the Carboxypeptidase Activity*

(Standard = 100)

Salts	Concentration of salts in the reaction mixture	Hydrolysis rate (*per cent*)			pH	Activation	Inhibition
		15 min.	30 min.	60 min.			
	M						
KCN	1×10^{-2}	63.6	60.4	67.2	7.56	−	++
	4×10^{-3}	84.7	90.4	94.8	7.32		
	2×10^{-3}	100.0	97.7	100.0	7.35		
$K_4Fe(CN)_6$	1×10^{-2}	106.7	109.6	112.1	7.32	+	−
	2×10^{-3}	105.1	102.2	108.6	7.44		
	1×10^{-3}	103.4	101.1	104.3	7.52		
$K_3Fe(CN)_6$	2×10^{-3}	144.1	124.5	139.1	7.40	++	−
	1×10^{-3}	118.3	105.3	117.2	7.35		
	2×10^{-4}	108.5	105.3	116.5	7.42		
$FeSO_4(NH_4)_2SO_4$	2×10^{-3}	110.0	107.4	110.3	7.40	+	−
	1×10^{-3}	100.0	100.0	101.7	7.43		
$NH_4Fe(SO_4)_2$	2×10^{-3}	94.8	98.9	98.2	7.43	−	−
	1×10^{-3}	93.2	96.8	100.0	7.36		
$Na_2Fe(CN)_5NO$	2×10^{-3}	103.4	102.1	103.4	7.35	+	−

tyrosine by means of carboxypeptidase was examined in relation to the initial rate of hydrolysis as well as to the optimum pH for the enzyme action.

1. NaCl, KCl, NaF, $MgCl_2$, $CaCl_2$, $BaCl_2$ and $AlCl_3$ exert no considerable influence on the action of carboxypeptidase.

2. Cobalt, nickel, ferrous and ferric salts accelerate the carboxypeptidase activity, the promoting effect being found to diminish in the following order: $Co^{++}>Ni^{++}>Fe^{++}>Fe^{+++}$. Manganese salt exhibits no influence on the enzyme.

3. Carboxypeptidase undergoes considerable inhibition by means of silver and mercury salts, while it is entirely unaffected by copper, zinc and lead salts.

4. KCN causes a marked inhibition of carboxypeptidase.

TABLE V

Influence of $KMnO_4$ and H_2O_2 on the Carboxypeptidase Activity

(Control=100)

Salts	Concentration of salts in the reaction mixture	Hydrolysis rates (*per cent*)			pH	Activation	Iahibition
		15 min.	30 min.	60 min.			
	M						
$KMnO_4$	5×10^{-4}	96.6	94.6	99.1	7.37	—	—
	2×10^{-4}	110.3	101.1	106.1	7.46		
	1×10^{-4}	101.7	100.0	101.0	7.38		
H_2O_2	5×10^{-3}	100.0	101.1	100.9	7.35	—	—
	2×10^{-3}	101.7	101.1	101.0	7.40		

5. Carboxypeptidase is accelerated by most of the iron complex salts tested, except in the case of ammonium ferricalum, which is found to have no effect.

6. Potassium permanganate and hydrogen peroxide exercise no effect on the enzyme.

7. All the salts tested caused no shift of optimum pH for the action of carboxypeptidase.

In conclusion the author wishes to express his sincere thanks to Prof. Dr. T. Uchino for his kind guidance throughout this research.

The expence of this research has been defrayed from the Scientific Research Expenditure of the Department of Eeucation.

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The Journal of Biochemistry, Vol. 40, No. 3.

STURUCTURE OF SPONGOTHYMIDINE

By KIYOO SATOH

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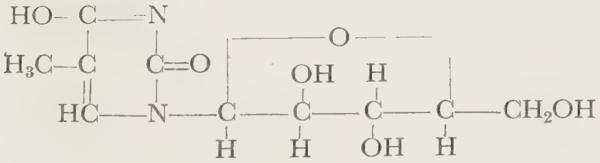
(Received for publication, March 19, 1953)

W. Bergmann and R. Feeney (*1*) have obtained a thymine pentosenucleoside from sponge of *Cryptotethia genus* and named it "spongothymidine." They have obtained thymine from this nucleoside by hydrolysing it with 10 per cent sulfuric acid. As to the structure of its sugar component they have suggested D-xylofuranoside from the results of the periodate titration, benzoylation and from the data of optical rotation. At our request Drs. Bergmann and Feeney have kindly sent us their sample. According to our investigation this nucleoside consumed one mole of periodate (*2*) without yielding formic acid agreeing with the description of Bergmann and Feeney but showed a negative Böeseken (*3*) boric acid reaction. So arabinose and ribose were excluded as the sugar component of spongothymidine. To settle the question for the sugar component concerns, arabinose or xylose, we made filterpaper chromatography with the sugar solution, which was prepared by hydrolysing the nucleoside with sulfuric acid. The Rf (0.301) of the sugar component coincided with that of xylose (Rf (0.303) in mixed chromatogram, but there was found no Rf valves corresponding those of arabinose (Rf 0.269), D-ribose (Rf 0.329) or D-2-desoxyribose (Rf 0.464). As the spongothymidine shows a relatively high dextrorotation, the sugar component must be D-xylose. Thence it follows that the structure of spongothymidine is D-xylofuranosylthymine (I).



(**I**)

EXPERIMENPAL

The Böeseken Boric Acid Test for the Corresponding Adjacent Hydroxyls—100 ml. of 0.05 *M* boric acid solution were made faintly red to phenolphthalein by the addition of 0.1 *N* sodium hydroxide solution (7.5 ml.).

In 0.4 ml. of dilute sodium hydroxide solution (2 drops of 0.1 *N* NaOH in 100 ml. of water), was dissolved the amount as in dicated in Table I of adenosine, glucose or xylose. The solution was just red to phenolphthalein. In the case of spongothymidine, uridine or cytidine one more drop of 0.01 *N* NaOH was added to the similarly treated alkali solution in order to gain the same red colour intensity. To make the dissolution complete each solution was warmed weakly. Each 0.1 ml. of borate solutions was added to all the test solutions. The results are shown in Table I. The solution of spongothymidine did not decolorize at all, while other solutions especially ribose-nucleosides showed complete decolorization immediately.

Table I

The Boeseken Boric Acid Test

Substrates		Results
	mg.	
Adenosine	1.38	⧻
Guanosine	1.42	⧻
Uridine	1.21	⧻
Cytidine	1.24	⧻
D-Glucose	0.90	⧻
D-Xylose	0.75	++
Spongothymidine	1.29	–
Control	—	—

Periodate Titration—To 1.290 mg. of spongothymidine were added 0.2 ml. of 0.05 *M* KIO_4 solution (F=1.009) and kept at room temperature for 10 hours and after addition of kalium iodate and sulfuric acid, back-titration was made with thiosulfate solution. 1.38 ml. of 0.05 *M* thiosulfate (F=1.016) were used. This shows that spongothymidine consumed 0.99 mol. of periodate without yielding formic acid.

Paper partition Chromatography of the Sugar Component—Solvent for the separation of sugars. normal butanol: glacial acetic acid:water=4:1:1.

Detecting reagent: Tollens reagent (ammoniakaline silver nitrate solution). As to the nucleosides the sugar solutions for chromatography were prepared as follows;

Adenosine and guanosine with 0.1 *N* H_2SO_4, and cytidine and spongothymidine were hydrolysed with *N* H_2SO_4 in sealed tube for 1 hour in the boiling water bath. The results are indicated in Table II.

TABLE II

Rf Values of Pentoses

Nucleoside	Sugar	Rf Value			Average
	D-Arabinose	0.267	0.267	0.272	0.2693
	L-Arabinose	0.270	0.266	0.273	0.2693
	D-Xylose	0.307	0.304	0.299	0.3000
		0.298			
Spongothymidine		0.301	0.303	0.302	0.303
		0.300	0.305	0.308	
	2-Desoxy-D-ribose	0.464			0.464
Cytidine	D-Ribose	0.330	0.330		0.330
Adenosine	”	0.330			0.330
Guanosine	”	0.330	0.330		0.330

The author wishes to thank Prof. Dr. Katashi Makino for his kind guidance. Aided by a grant from the Scientific Research Fund of the Department of Education (Katashi Makino).

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FURTHER STUDIES ON THE BINDING OF DYES BY PROTEINS

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(Received for publication, March 18, 1953)

There are a large number of reports so far published (*cf.* (*1*) and its references) concerning the binding of acidic dyes by serum proteins. The author reported in the previous paper (*2*) the results of his studies on the binding of methyl orange and phenol red by serum albumin and discussed the relation between the dye binding ability and the spatial configuration of native and denatured proteins. It was mentioned there that, among serum proteins, only albumin combined with methyl orange and its dye-binding ability decreased by denaturation of the protein, and also that neither γ-globulin nor albumin combined with basic dyes such as toluidine blue, neutral red, etc., at the concentrations of the dyes where they were thought to be monodisperse. Bennhold (*3*), Goldstein (*4*) Klotz (*5*), and others also studied the binding of basic dyes, other than mentioned above, by serum proteins and stated that the dyes did not combine with the proteins.

The present paper deals with the binding of basic dyes by proteins at higher concentrations of the dyes and the alteration in dye binding ability of serum albumin and γ-globulin by denaturation of the proteins. The behavior of Bence-Jones protein was also examined.

EXPERIMENTAL

Bovine serum albumin, γ-globulin, and urinary Bence-Jones potein from a myeloma patient were used as proteins, bromophenol blue (tetrabromosulfophthalein, B.P.B.) as an acidic dye, and toluidine blue (T.B.), methylene blue (M.B.), and rosaniline hydrochloride (R.H.) as basic dyes.

Crystalline bovine serum albumin was prepared by Kekwick's method (6). Bovine serum γ-globulin was precipitated by salting-out method from the whole serum with sodium sulfate at the concentration of 14.5 g./dl., the precipitate was dissolved in water and dialyzed against 0.9 per cent sodium chloride. Purity of the protein preparations used was above 90 per cent in all cases.

To prepare denatured proteins, 0.1 *N* NaOH was added to 2—3 g./dl. native protein solutions to the concentration of 0.03 N (pH 12.0). At various time intervals after the addition of NaOH, the solutions were neutralized with H_2SO_4 so as to bring pH of the neutralized solutions to 6.7.

Bence-Jones protein was prepared from urine of a patient suffering from myeloma by salting-out method with ammonium sulfate. It had the same solubility characteristics as those reported in literatures.

As dyestuffs Merck's or Kahlbaum's preparations were used with or without recrystallization.

Spectrophotometric determination of the bound dyes was carried out according to the procedure published by Shimao (*7*).

RESULTS

Binding of T. B., and R. H. by Native Serum Albumin and γ-Globulin—According to Michaelis (*8*), T. B. and M. B. have absorption maximum (α-peak) at 640 mμ and 670 mμ, respectively, and at higher concentrations a new absorption maximum (β-peak) appears in the range of shorter wave length. The wave length of the maximum of the new absorption is 590—600 mμ for T. B. and 610 mμ for M. B. These new absorption maxima are those due to polymerized dyes and they can be reduced or even extinguished by the addition of yeast nucleic acid which combines with the dyes and reduces their effective concentration. The author also confirmed these facts.

The spectral absorption curves of the mixed solutions of each of these dyes and native albumin or γ-globulin were found to be identical with those of the pure solutions of the dyes. The concentrations of the dyes used in these experiments were 2.64×10^{-4} M for T. B., and 6.70×10^{-5} M for M. B., and at these concentrations the absorption curves of both of the dyes showed both α- and β-peaks. The absorption curve of monodisperse R. H. showed a maximum at 540 mμ and no new absorption maximum appeared in this curve by the increase of the dye concentration. This dye also showed no remarkable change in the absorption curve by the addition of native proteins at any protein concentration and pH tested (pH 5.9 and 6.5).

The above results indicate that there is no interaction between the basic dyes and the native proteins examined.

Binding of T. B. and M. B. by Denatured Serum Albumin and γ-Globulin—When the denatured serum albumin or γ-globulin was added to the solution of T. B. or M.B. whose absorption curves showed both α- and β-peaks, the following facts were observed. In the case of T. B., the height of the α-peak increased by the addition of either albumin or

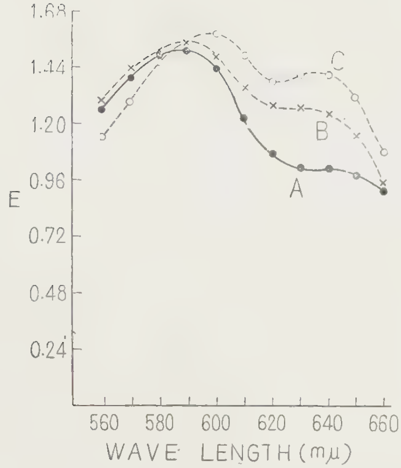

Fig. 1. Absorption spectra of T.B.
A: in 0.03 N Na_2SO_4
B: in the presence of the alkali-treated bovine serum albumin
C: in the presence of the alkali-treated bovine serum γ-globulin
pH: 6.9, Concentration of T.B.: 2.64×10^{-4} M,
Concentration of protein: 0.4%, in all cases

γ-globulin, and the effect of the addition of γ-globulin was more distinct than that of albumin at the same protein concentrations (Fig. 1). In the case of M. B., on the other hand, the absorption curve showed some change only when the denatured γ-globulin was added (Fig. 2). Both T. B. and M. B. showed no apparent interaction with the denatured proteins at lower concentrations where the absorption curves of the dyes showed no β-peak.

Binding of R. H. by the Denatured Serum Albumin and γ-Globulin—The solution of R. H. follows Beer's law at concentrations below 2.70×10^{-5} M. Accordingly, the solution of R. H. seems to be monodisperse at the range of such concentrations. The addition of the denatured γ-globulin, but not that of the denatured albumin, induced change both on E′ (*cf.* (*7*)) and the absorption curve of the dye. The change thus induced increased with the time interval of alkali-treatment of the γ-globulin (Table I).

Binding of B.P.B. by Serum Albumin and γ-Globulin—B.P.B. was used by Durrum (*9*) as a staining agent in the study of electrophoresis on filter paper. It has an absorption maximum at 590 mμ and when serum albumin was added to the dye solution, its color changed as the result of a shift of the absorption maximum to 600 mμ.

n-Values of serum albumin at pHs 5.6, 6.0, 7.0, and 8.0, were 6.0, 5.7, 5.0, and 4.0, respectively. As shown by the above figures, *n*-value of serum albumin decreased when pH changed from acid to alkaline side. In the presence of 2.9 per cent fomaldehyde, at pH 6.7, the bound B.P.B. decreased to 63 per cent of the original value. These tendencies were quite the same as those found in the case of the binding of methyl

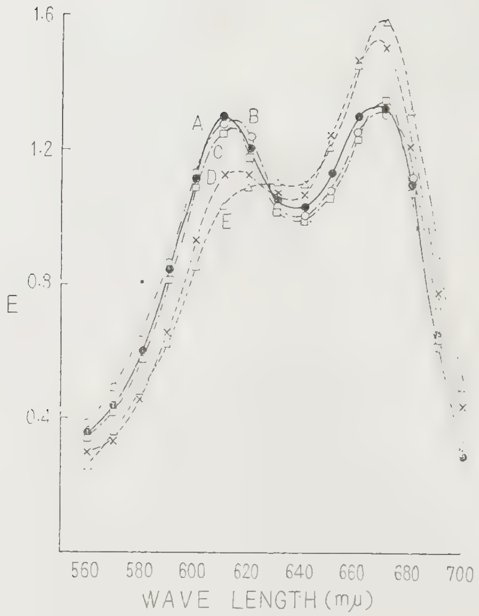

FIG. 2. Absorption spectra of M. B.

A: in 0.03 N Na_2SO_4
B: in the presence of the alkali-treated bovine serum albumin (1.0%)
C: in the presence of the alkali-treated bovine serum albumin (0.4%)
D: in the presence of the alkali-treated bovine serum γ-globulin (0.4%)
E: in the presence of the alkali-treated bovine serum γ-globulin (0.5%)
pH: 6.9. Concentration of M.B.: 6.70×10^{-5} M, in all cases.

orange (M.O.) by serum albumin.

While dye-binding ability of serum albumin decreased by alkali denaturation of the protein, γ-globulin showed dye-binding ability only after alkali-treatment (Table II). Furthermore, it was found that the longer the time interval of alkali-treatment, the more the amount of bound dyes. Change in the absorption curve induced by the addition of the denatured γ-globulin had the same tendency as that induced by serum albumin.

Binding of M. O. and R. H. by Bence-Jones Protein—As can be seen from Table III, Bence-Jones protein showed appreciable interaction with R. H., but no interaction with M. O. when the protein was heated

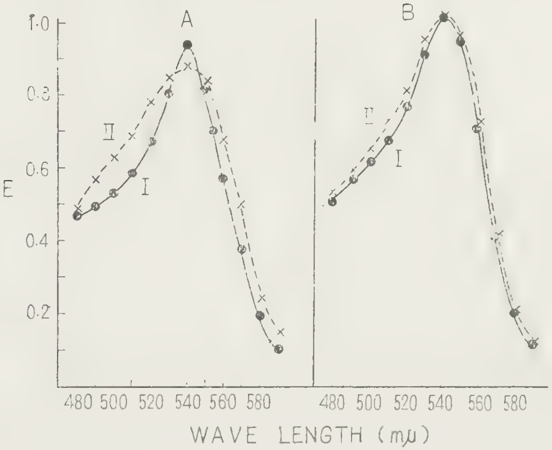

Fig. 3. Absorption spectra of R.H.

A; I: in phosphate buffer at pH 6.6

II: in phosphate buffer containing the alkali-treated γ-globulin at pH 6.6.

B; I: in phosphate buffer at pH 6.0

II: in phosphate buffer containing the alkali-treated γ-globulin at pH 6.0

Concentration of R.H.: 1.28×10^{-5} *M*. Concentration of protein: 0.26%, in all cases.

Table I

Characteristics of the Binding of R. H. by the Alkali-denatured γ-Globulin

(pH 6.5, *M*/15 phosphate buffer)

Treatment	Temperature of determinatiun	$K\times10^{6}$	$k_1\times10^{-6}$	ΔF_1	n
				cal./mole	
0.03 *N* NaOH, 37°, 1 hr.	16°	1.28	2.46	−8460	3
„ „ , 37°, 24 hrs.	13°	1.68	3.14	−8500	5
„ „ , 14°, 40 hrs.	14°	1.08	4.80	−8760	5
„ „ , 37°, 40 hrs.	14°	1.76	3.94	−8660	7

K: Intrinsic dissociation constant,

k_1: First association constant,

ΔF_1: $-RT \ln (k_1)$,

n: maximum binding number per molecule.

Moleculer weight was taken as 156,000 for γ-globulin.

TABLE II

Characteristics of the Binding of B.P.B. by Proteins

(pH 6.7, *M*/15 phosphate buffer)

Material	Treatment (0.03 *N* NaOH)	Temperature of determination	$K \times 10^6$	$k_1 \times 10^{-6}$	ΔF_1	n
					ca./mole	
Bovine serum albumin	(native)	14°	1.21	3.92	−8660	5
	14°, 1 hr.	16°	0.678	5.46	−8910	4
	37°, 1 hr.	16°	2.66	1.20	−8060	3
	14°, 24 hrs.	15°	2.55	1.31	−8070	3
	37°, 24 hrs.	15°	2.79	0.83	−7920	2
	14°, 40 hrs.	13°	1.05	2.10	−8280	2
	14°, 70 hrs.	14°	1.23	1.63	−8170	2
Bovine serum γ-globulin	37°, 1 hr.	16°	0.780	2.37	−8440	2
	14°, 24 hrs.	13°	0.877	2.96	−8460	3
	37°, 24 hrs.	13°	0.446	7.18	−8990	3
	14°, 40 hrs.	14°	1.09	3.03	−8520	3
	37°, 40 hrs.	14°	3.32	1.14	−7950	4

K, k_1, ΔF_1, and n; (Same as in Table I.)

Molecular weight was taken as 69,000 for albumin and 156,000 for γ-globulin.

at 100° for 15 minutes and then cooled to the room temperature, E′-value of the protein decreased to one-half of the original value in spite of the perfect recovery of solubility.

DISCUSSION

As can be seen from the foregoing results, basic dyes such as T. B., M. B., and R. H. do not show any appreciable interaction with native bovine serum albumin and γ-globulin even at high concentrations of the proteins, but readily interact with alkali-denatured γ-globulin. Moreover, while B.P.B.-binding ability of serum albumin decreases by alkali-treatment, that of γ-globulin appears only after alkali-treatment of the protein. Free energy changes of the binding are about 8.5 *Kcal./mole* in all cases (Tables I and II), and these values are larger than the value for the binding of M. O. by serum albumin. It seems, therefore, that the bonds between the proteins and the above dyes are relatively

TABLE III

Binding of Dyes by Bence-Jones Protein

(pH 6.5, *M*/15 phosphate buffer)

	Dye	Dye concentration	Protein concentration	E′
		M	*per cent*	
Before heating	M.O.	5.10×10^{-5}	0.2	0.003
After heating	,,	,,	,,	0.004
Before heating	R.H.	2.48×10^{-5}	0.2	0.170
After heating	,,	,,	,,	0.081

strong. According to Michaelis (*8*) and Cavalieri, *et al.* (*10*), these basic dyes also combine with pentose- and desoxypentosenucleic acids at the phosphate radicals of the latter.

It is necessary to study pH-dependence of the dye-binding ability for the determination of the binding sites of the proteins, but it was impossible in the present experiment owing to the change of the solubility of the proteins. Nevertheless, from the fact that alkali-treated γ-globulin showed no interaction with R. H. at pH 6.0 but some interaction with R. H. at pH 6.6, as shown by Fig. 3, and also from my previous observation that acidic protein, pepsin, interacted with R. H. even in the native state, it would seem certain that interaction occurred between amino groups of the basic dyes and the free acidic groups of the proteins. The binding of B.P.B. by the protein seems to occur between sulfonic group of the former and free basic groups of the latter, from the fact that the binding characteristic was nearly the same as that of M. O. reproted previously.

Not only net charge of a protein molecule but also relative position of free acidic and basic groups and other various polar groups on the surface of the protein molecule take part in the binding of dyes. It seems, therefore, that native albumin molecules have more free basic groups than acidic groups and that these basic groups are pertinently situated so as to be able to bind dyes. In the alkali-denatured state it seems that the basic and acidic groups are bound to each other resulting in the loss of free groups and thus the decrease is brought about in dye-binding ability for both acidic and basic dyes. For the same reason, native γ-globulin seems to have acidic and basic groups bound to one another intramolecularly and these groups are partically liberated on the surface of the molecules by alkali-treatment, resulting in the acquisition of the dye-binding ability.

Further studies by various other investigation methods are, of course, necessary for the decisive determination of the intramolecular arrangement of these kinds of bonds.

Van der Scheer (*13*) and Hirai and Shimao (*11*) in this laboratory reported that, when alkali-denatured serum was studied electrophorecically, three globulin peaks of native serum were converged into one peak having a mobility between that of native α- and β-globulins. Hirai (*11*) also showed by Willstaetter's titration method that the increase in carboxyl group by alkali denaturation was more remarkable with γ-globulin (160 per cent increase) than with albumin. These facts, together with the results reported in this paper, indicate that negatively charged groups in γ-globulin are released by alkali-denaturation and take proper configuration for the binding of basic dyes.

Bence-Jones protein has well-known characteristic solubility, but its physico-chemical properties have not been studied in detail because of the rare appearance of the patients. This protein is said to have the mobility between those of β- and γ-globulins when studied electrophoretically at pH 8 to 8.6 (*14*). While all the globulin fractions of normal human serum failed to bind R. H., Bence-Jones protein bound it as mentioned above, and in this respect this protein has different properties from serum globulins. Notwithstanding the perfect reversibility of heat denaturation of the protein when tested by solubility, its dye-binding ability changed after heat-treatment, indicating that its molecular structure suffered change by the treatment.

SUMMARY

1. Both bovine serum albumin and γ-globulin did not bind T. B. and M. B. at high concentrations of the dyes. After alkali-treatment of these proteins, only denatured γ-globulin bound T. B. and M. B. remarkably resulting in the depolymerization of the dyes.

2. R. H. was bound by alkali-treated γ-globulin but not by albumin at the concentrations of the dye where it obeyed the Beer's law.

3. B.P.B.-binding ability of native serum albumin was lost by alkali-treatment of the protein, while, in the case of γ-globulin, the dye-binding ability appeared only after alkali-treatment.

4. Bence-Jones protein prepared from urine of a myeloma patient had the ability to bind R. H., and this ability decreased after heat-treatment, while its solubility did not change at all.

The author expresses his thanks to Prof. N. Shimazono for his support and advice, and also to Dr. H. Hirai and K. Shimao for their help throughout these investigations.

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STUDIES ON XANTHURENIC ACID. I.

THE EFFECT OF FATTY ACID ON THE EXCRETION OF XANTHURENIC ACID, AND ITS RELATION TO PYRIDOXINE

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(Received for publication, March 23, 1953)

In 1935, Musajo (*1*) fed white rat with fibrin and isolated a yellow crystal compound which he named xanthurenic acid. Later this compound was confirmed as 4,8-dihydroxyquinoline-2-carboxylic acid (*2*) and derived from tryptophan passing kynurenin in vivo (*3*, *4*). In 1943, Lepkovsky, Roboz and Haagen-Smit (*5*) noticed the appearance of a large amount of xanthurenic acid in the urine of a white rat, which was placed on a pyridoxine deficient but tryptophan rich diet for a long time, and also it disappeared quickly when pyridoxine was given. They succeeded to isolate xanthurenic acid from this urine relatively easily by the help of a paper packed chromatographic column (*9*). This finding led them to conclude that xanthurenic acid is an abnormal metabolite of tryptophan when the body is pyridoxine deficient. Further this compound was found in the urines of rabbit, dog, guinea pig, pig, human being (*7*, *8*) *etc.*, but not in that of chickens.

We accidentally came to produce pyridoxine deficiency in white rat very simply in a short time by giving sodium salt of fatty acid orally, and succeeded in easily isolating xanthurenic acid in a crystal form by giving tryptophan at the same time and collecting the 24 hours urine. The experimental procedures will be given in this report.

EXPERIMENT

White rat of a body weight 120–150 g. was made hungry for one day, and given sodium butyrate 0.3 g. and tryptophan 0.1 g. orally at the same time. After several hours was excreted a thick yellow urine, which gave a remarkable green color reaction due to xanthurenic acid by adding a minute amount of ferric iron. From the urines, which were obtained by collecting 24 hours urines of 10 white rats we obtained xanthurenic acid in crystal form by using the paper chromatographic method of Lepkovsky *et al.* The yield was 30 mg. The ferric iron reaction, the diazo reaction, the Millon reaction and the HCl- and $KClO_3$ reaction all came out positive with the obtained sample.

The Effect of Fatty Acids on the Excretion of Xanthurenic Acid—We compared the amounts of xanthurenic acid in the urines of white rats when 0.4 g. of Na-salts of various fatty acids were given at the same time with 0.1 g. of tryptophan. The xanthurenic acid was measured colorimetrically by Miller and Baumann's method (*9*). The results are shown in Table I.

TABLE I

The Effect of Fatty Acid Salts

Sorts of Na-salts of fatty acids	Xanthurenic acid
	mg.
Tryptophan only	1.61
Acetate	5.37
Propionate	8.79
Butyrate	9.89
Valerianate	9.64
Palmitate	9.61
Stearinate	8.57
Oleate	10.49

It is apparent from Table I that more xanthurenic acid is excreted when fatty acid is administered with tryptophan. In this case butyric acid is more effective than acetic and propionic acids, but any fatty acid higher than butyric seems to have nearly the same effect as butyric.

The Same Experiment in the Presence of Pyridoxine—From the fact that more xanthurenic acid was excreted in the urine when fatty acid was administered with tryptophan we assumed that pyridoxine storage became acutely short in the body of the white rat when fatty acid was administered. One of the symptoms of pyridoxine deficiency is the browning of hair, which was really confirmed in this case. If so, when pyridoxine is administered together with sodium butyrate and tryptophan the amount of xanthurenic acid should be reduced. This is true as shown in the following table.

SUMMARY

1. When Na-salts of fatty acids were administered to rat together

TABLE II

The Effect of Fatty Acid Salt in the Presence of Pyridoxine

Sorts of compounds administered	Xanthrenic acid
	mg.
Tryptophan plus sodium butyrate (0.3 g.)	8.42
Tryptophan plus sodium butyrate (0.3 g.) plus pyridoxine (injection) (10 mg.)	2.03

with tryptophan the excretion of xanthurenic acid into urine was greatly increased.

2. This fact should be ascribed to the used-up of pyridoxine for the oxidation of fatty acid, as the injection of pyridoxine checked such an increase.

We wish to thank heartily to Prof. Yashiro Kotake, for his advice and ec-couragement to this research.

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STUDIES ON XANTHURENIC ACID. II.

PRELIMINARY REPORT ON XANTHURENIC ACID DIABETES

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(Received for publication, March 23, 1953)

Since the diabetogenic activity of alloxan was reported, the question of its biological origin and its possible relationship to the etiology of diabetes mellitus has been much discussed. From its structural similarity to uric acid it is postulated that alloxan might be produced in the body by oxydation of uric acid (*1*). But no decisive proof has been raised. Meanwhile, in this country Okamoto *et al.* who discovered that the cells in island of Langerhans are rich in zinc, reported that diabetes can be produced experimentally by administering reagents such as oxine, dithizone or nicotin which have strong affinity to zinc (*2*, *3*, *4*). At present oxine, dithizone and nicotin etc. are considered irrelevant to metabolism.

But these chemical substances can not be considered to be naturally occuring during metabolic processes in animal body. In this concern we happened to discover that diabetes can be produced by xanthurenic acid, which was first claimed by Lepkovsy (*5*) to be a metabolite of tryptophan in vitamin B_6 deficiency, and also by us to be abundantly formed in animal body when tryptophan is given with fatty acid together (*6*). As both substances occur in our daily diets and especially rich in luxurious one, it is quite interesting to investigate to what extent xanthurenic acid is responsible in etiology of human diabetes.

EXPERIMENTSL

To isolate xanthurenic acid, as previously reported, we administered to rat *per os* sodium butyrate (0.4 g.) and tryptophan (0.1 g.) several times, and after feeding about 150 days we accidentally found that cataract occurred in 3 rats among 40. This finding led to the idea that xanthurenic acid might be an etiologic factor of diabetes in animal. So we determined blood sugar of rats, one group of which was fed on the basal diet together with sodium butyrate and tryptophan, and another group

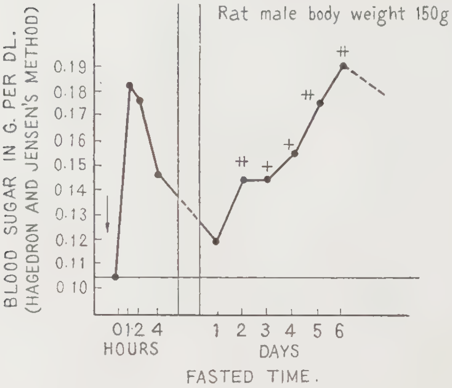
Rat male body weight 150g
BLOOD SUGAR IN G. PER DL.
(HAGEDRON AND JENSEN'S METHOD)
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
01 2 4
HOURS
1 2 3 4 5 6
DAYS
FASTED TIME.

Fig. 1.

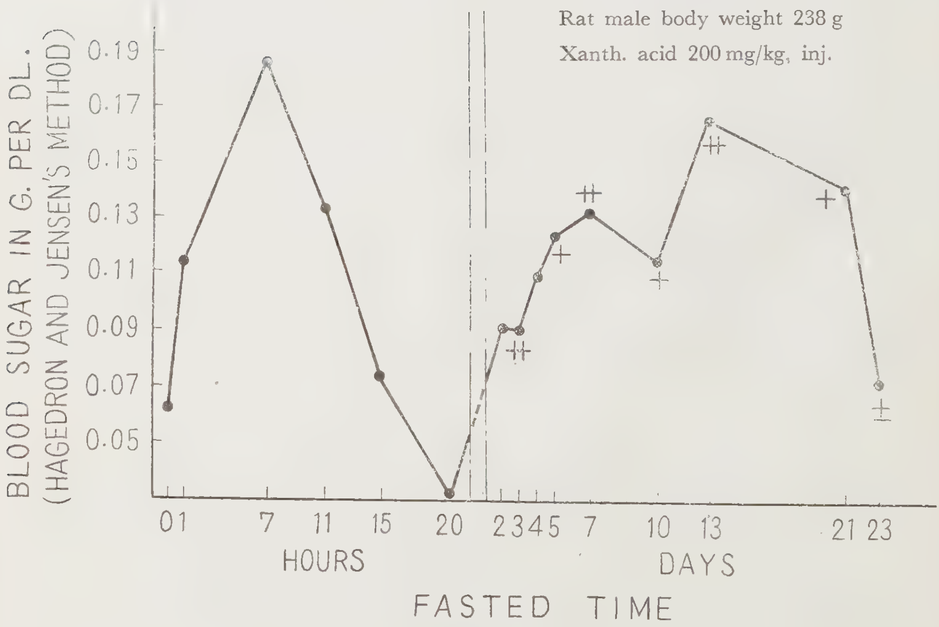
Rat male body weight 238 g
Xanth. acid 200 mg/kg, inj.
BLOOD SUGAR IN G. PER DL.
(HAGEDRON AND JENSEN'S METHOD)
0.19
0.17
0.15
0.13
0.11
0.09
0.07
0.05
0 1 7 11 15 20
HOURS
2 3 4 5 7 10 13 21 23
DAYS
FASTED TIME

Fig. 2.

was injected with water solution of natural xanthurenic acid crystal into interperitoneal cavity. The basal diet was composed of casein 22 per cent, starch 52 per cent, yeast 2 per cent, agar 3 per cent, Mc Collum salt mixture 6 per cent, butter 10 per cent and sucrose 5 per cent. Typical experimental results are illustrated in Figs. 1 and 2.

In all cases of 30 rats without exception, initial hyperglycemia occurred about one hour after administration, followed by passing hypoglycemia and then again notable hyperglycemia which continued for several days.

In parallel to hyperglycemia, glycosuria occurred and the animals tended to consume more foods and water.

Histologic examination.

Some remarkable changes were observed histologically on the rat pancreas. The rat was injected intraperitoneally with 200 mg. xanthurenic acid *pro kilo* body weight, and 48 hours later the animal was sacrificed, the pancreas was taken out, and subjected to histological preparate as usual, which was stained by Gomori's chrome alum hematoxylin phloxin method (8, 9). It was found that the cytoplasma in the greater part of beta cells of islands of Langerhans stained not so good, became transparent and some of them vacuolized. Further in a few beta cells, the nuclei conglomerated and fell into pycnosis. No changes were seen in alpha cells. (The examination was done with the help of the anatomy and pathology departments of our medical college.)

SUMMARY

Though it is necessary of further precise experiments to get the final conclusion yet the findings so far attained such as changes of pancreas, the occurrence of cataract and hyperglycemia may justify the conclusion that xanthurenic acid is responsible for the production of pancreatic diabetes. The structual similarity of xanthurenic acid to oxine, which is proved by Okamoto to be diabetogenic, also favour the above postulation. Since xanthurenic acid, however, is more common metabolites in the living body than alloxan or oxine, we think that our finding give s an important clue to the etiology of human diabetes.

We wish to express our deep gratitude to Dr. Yashiro Kotake who has given very kind guidance in carrying out this research.

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STUDIES ON XANTHURENIC ACID III.

XANTHURENIC ACID IN THE URINE OF DIABETIC PATIENT

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(Received for publication, March 23, 1953)

Xanthurenic acid was discovered in 1935 by Musajo (*1*) in the urine of white rat and then made clear by him that it originated in tryptophan (*2*, *3*). Then, it was noticed by Lepkovsky *et al.* (*4*) that it is formed in greater amount from tryptophan in animal body when pyridoxine is deficient.

One of the authors (*5*) succeeded in isolating xanthurenic acid more efficiently from the urine of white rat by administering sodium salt of fatty acid and tryptophan together.

Further, it was noticed by them (*5*) that diabetic symptoms appear in white rat after administering sodium salt of fatty acid and tryptophan together, and also by administering natural xanthurenic acid, isolated from white rat urine, or synthetic xanthurenic acid (m.p. 295.5°, synthesized by Takeo Sakan).

From these results, it is postulated that rat is led to acute pyridoxine-deficiency by taking a large amount of fatty acid (fat), and thus fells into an abnormal metabolism of tryptophan, producing xanthurenic acid, which acts as a factor responsible for diabetic symptoms of rat. It is also postulated that the finding may have some important relation to diabetes mellitus in human being. As a matter of fact, diabetic patients are more common among the inhabitants of a civilized country where a large amount of fat and animal protein containing much tryptophan are more abundantly consumed.

To raise an additional evidence on this point, the authors examined whether xanthurenic acid can be found out or not in the urine of diabetic patients.

For this purpose we applied paper chromatography and examined the urine of 8 cases of diabetes patients with the result that surely the excretion of xanthurenic acid occurred in every case. Further we happened to prove the existence of 3-hydroxykynurenine which is considered as the precursor of xanthurenic acid.

EXPERIMENT

Eight cases of diabetes patients under treatment in the hospital of the Wakayama Medical College and 2 cases of healthy persons as control were taken for examination. The patients were all fairly sever and treated with 20–30 units of insulin every day.

The urines during 24 hours were collected, reduced to small volume as possible, filtered, extracted and, if necessary, alcohol was added to the concentration of 50–60 per cent filtered. The filtrate was freed of alcohol by evaporation and treated and with mercuric acetate. The precipitate was suspended in water and completely decomposed by H_2S. The filtrate was concentrated to about 5 ml. in vacuum, and then submitted for paper chromatography.

The paper was No. 2 of Toyo filter paper and the developing reagent was the mixture of butanol, acetic acid and water, weight proportion 4:1:1, the developing interval being 15 hours.

EXPERIMENTAL RESULTS

The results on healthy urine (A) and on the mixture of kynurenine, 3-OH-kynurenine and xanthurenic acid (C) for the sake of comparison were illustrated in Fig. 1 with the result on diabetic urine (B). 8 cases of diabetic urine showed the same developement. Fig. 1.

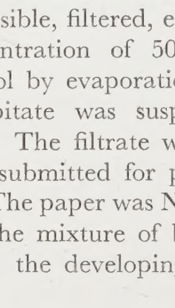

Fig. 1

A: Healthy urine
B: Patient urine
C: Mixture of kynurenine, isolated from rabbit urine administered tryptophan, synthetic 3-OH-kynurenine and xanthurenic acid

In the figure only the spots were shown, which existed in common in all 8 cases, and were judged as the same substances from Rf value, or from the colour of fluorescence. Some physical and chemical properties of each spot are indicated in Table I.

It will be acknowledged from the above results that the spot F_5 of diabetic urine, corresponding to X of C, is due to xanthurenic acid. Further, F_4 coincides with kynurenine (K of C), F_3 with 3-OH-kynurenine (3-OK of C). F_2 and F_1 are judged according to the description of A. Neuberger *et al.* (*6*), probably to be con-

TABLE I

Colour reaction	A			B				C		
	F_1'	F_2'	F_3'	F_1	F_3	F_4	F_5	3-OK	K	X
Rf value	0.07		0.4	0.06	0.32	0.41	0.5	0.32	0.41	0.5
Bratton-Marshall	−		+	−	−	+	−	−	+	−
Ehrlich	+		−	+	+	−	+	+	−	+
Pauly	+		−	+	+	−	+	+	−	+
Millon	−		−	−	−	−	+	−	−	+
Fe^{+++}	−		−	−	−	−	+	−	−	+
Ninhydrin	+		+	+	+	+	−	+	+	−

jugated derivatives of xanthurenic acid and 3-OH-kynurenine, respectively. On the 2 cases of healthy normal urine, F'_3 corresponding to kynurenine was noticed and no spot corresponding to free 3-OH-kynurenine or xanthurenic acid was noticed. But though very slightly, the spots corresponding to conjugated derivatives of xanthurenic acid or of 3-OH-kynurenine as F_2' and F_1' were detected.

This fact shows that even in healthy urine, minute amount of 3-OH-kynurenin and xanthurenic acid comes out exclusively in conjugated form, while in diabetic patient urine the substances appeared in free form. The experiment was repeated with diabetic urine, to which a small amount of synthetic xanthurenic acid crystal was added. It was found in this case that the fluorescence and other colour reactions of the spot F_5 (B) were remarkablly enlarged and intensified. Quite similar results were obtained on the urines, to which kynurenine and 3-OH-kynurenine were added.

SUMMARY

The existence of xanthurenic acid and 3-OH-kynurenine in the urine of diabetes patient were confirmed. Taking into consideration the fact that xanthurenic acid causes diabetic symptoms in white rat, it will be postulated that xanthurenic acid also is a most plausible agent in pathogenesis of human diabetes.

We thank very much to Dr. Yashiro Kotake who encouraged and adviced us throughout this experiment and also to Dr. Takeo Sakan who was kind enough to donate us synthetic xanthurenic acid and 3-OH-kynurenine.

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